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Development of a strain-specific quantitative method for monitoring *Pseudomonas fluorescens* EPS62e, a novel biocontrol agent of fire blight

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Abstract

Pseudomonas fluorescens EPS62e has been selected in a screening procedure for its high efficacy controlling *Erwinia amylovora* infections in flowers, immature fruits and young pear plants. We developed two monitoring methods which allowed specific detection and quantification of EPS62e by combining classical microbiological techniques with molecular tools. RAPD and unspecific-PCR fingerprints were used to differentiate EPS62e from other *P. fluorescens* strains. Differential amplified fragments from EPS62e were sequence characterized as SCAR markers and two primer pairs were designed and selected for their specificity against EPS62e. A SCAR primer pair was evaluated and validated for the assessment of population dynamics of EPS62e on pear plants under greenhouse conditions using plating and most probable number assays coupled to PCR. Both techniques were useful in monitoring the biological control agent. The population level of EPS62e after treatment was 7 log CFU (g f.w.)$^{-1}$, which in turn decreased progressively to 4–5 log CFU (g f.w.)$^{-1}$ after 17 days and then remained stable until the end of the assay 11 days later. The limit of detection of both monitoring methods developed was around 3 log CFU (g f.w.)$^{-1}$, thus, providing a reliable tool for the analysis of EPS62e in greenhouse or field trials, and the assessment of threshold population levels for efficient biocontrol of fire blight.

Keywords: Fire blight; Biocontrol; Monitoring; PCR; *Pseudomonas fluorescens* EPS62e; RAPD; SCAR

1. Introduction

Erwinia amylovora is the causal agent of fire blight, a severe disease that affects rosaceous plants and causes great economic losses in pear, apple and ornamental plant production [1,2]. The main type of disease control is based on chemical bactericides, such as copper derivative compounds or certain antibiotics, depending on the specific regulations of each country [3]. Nevertheless, the selection of strains resistant to antibiotics, the environmental impact of such substances and consumer concerns about pesticide residues in food have favoured the development of alternative or complementary methods for disease management such as biological control [4–7].

Biological control against fire blight has been developed based on epiphytic bacteria [8,9]. Some strains belonging to different bacterial species have shown their aptitude in preventive application, such as *Pseudomonas fluorescens* A506 [8], *Pantoea agglomerans* C9-1 [10] or *Bacillus subtilis* QST 713 [11].

Since colonization is one of the factors involved in the mechanisms of action of many biological control agents, it is necessary to develop monitoring methods which

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allow the unambiguous identification of the strains and their quantification after field application [12]. Furthermore, *P. fluorescens*, *P. agglomerans* and *B. subtilis* are normal inhabitants of plants, so the method of analysis required must be able to discriminate at strain level [13].

Microbiological monitoring methods have been based on culture on selective media (e.g., supplemented with antibiotics) using plate counting or most probable number (MPN) methods. These techniques have the advantage of quantifying only viable cells and they do not require any special equipment. However, the high level of specificity required for strain traceability is barely reached by these microbiological methods. Moreover, antibiotic-marked strains, by means of gene insertion or spontaneous variants of the wild type, may present pleiotropic effects on its fitness [14] and population level may be overestimated because other bacteria present in the natural samples could be resistant to the same antibiotic used [15–17]. In the case of gene insertion, the strain becomes a genetically modified microorganism (GMM) and could be considered hazardous when released into the environment [18]. In order to improve microbiological monitoring methods, they can be combined with molecular techniques such as PCR.

Molecular monitoring methods based on the PCR technique using natural genomic markers of the wild type biological control agent, have largely enhanced the discrimination at strain level, thus minimising the drawbacks of introduced markers. Nevertheless, molecular markers may not be available when the genome of the species to be tracked is not well known, which often happens with many biological control agents. Then, natural polymorphisms can be detected by the use of random amplified polymorphic DNA (RAPD) [19,20] which does not require prior knowledge of the genome. RAPD has been commonly used for fingerprinting biological control agents [21–24]. Apart from RAPD which uses single primers, a variation of RAPD but this time using unspecific primer pairs (U-PCR) can be used for fingerprinting. The main differences between RAPD and U-PCR are found in the sequence's length and the primer design, being longer (~20 bp) and not randomized in the case of U-PCR.

Several authors have developed sequence characterized amplified regions (SCAR) as molecular markers [25] using one of the discriminatory fragments from a RAPD profile in order to design a specific primer pair for a biological control agent's detection. SCAR markers have been developed to detect the biological control agents *Trichoderma hamatum* 382 [26], *Clonostachys rosea* GR5 [27], *P. fluorescens* 29A [28], *Colletotrichum coccodes* 183088 [29], *Pichia anomala* K [30] and *Aureobasidium pullulans* L47 [31]. A PCR method, based on a molecular marker sequence of the spore coat protein, has been used for detection of the fire blight biocontrol agent *B. subtilis* DB170 [32]. However, this PCR method was not proven to be specific at strain level and was not coupled to a quantitative method. As far as we know there is no *P. fluorescens* biological control agent of fire blight for which a strain specific PCR based technique coupled to quantitative method has been developed.

Pseudomonas fluorescens EPS62e was selected among 600 strains of *P. fluorescens* and *P. agglomerans* because it exhibited effective control, mainly by competitive exclusion, of *E. amylovora* [33]. The development of this strain as a biopesticide for fire blight control would require specific analytical methods for monitoring and quantification of the strain in the environment.

Therefore, the aim of this work was to develop and compare two mixed monitoring methods for *P. fluorescens* EPS62e: the plating method with randomly picked colonies tested by PCR and the Most Probable Number-PCR method. We developed the RAPD and U-PCR profiles to discriminate EPS62e from other *P. fluorescens* strains and then the selected amplified fragments were sequence characterized as SCAR markers. SCAR primer pairs were designed and tested for their specificity and sensitivity in EPS62e detection by PCR against other strains and natural samples. Then two microbiological methods for monitoring EPS62e (Plating and MPN) attached to a PCR with SCAR primers were compared and used for the assessment of EPS62e population dynamics on the leaves of pear plants under greenhouse conditions.

2. Materials and methods

2.1. Bacterial strains and growth media

Strains used in this study are listed in Table 1. Bacteria were cultured at 25 °C on Luria-Bertani agar (LB) over 24 h. Long-term storage was carried out in a 20% glycerol nutrient broth at −80 °C.

EPS62e was characterized phenotypically and genotypically as a *P. fluorescens*. This strain did not present ice nucleation activity, nor did it induce a hypersensitive reaction on tobacco and geranium. It did not produce known antibiotic compounds such as 2,4-diacetylphloroglucinol, phenazine-1-carboxilic acid, pyrrolnitrin or hydrogen cyanide, nor plant growth regulators such as indol-3-acetic acid described in some strains of *P. fluorescens*. The 16S rDNA and the internal transcribed spacer (ITS) were sequenced. The sequence had high homologies to other 16S rDNA and ITS sequences of *P. fluorescens* strains from the GenBank Database, and finally, it was deposited with the Accession No. AJ583090.

2.2. Plant material and efficacy assays

Immature pear fruits (cultivar Passe Crassanne) and pear flowers (cultivar Doyenne du Comice) were

Table 1
List of bacterial strains used in this study

Species	Code	Origin[a]
Erwinia amylovora	662, 665	UPN
E. amylovora	EPS101, EPS102	UdG
Pantoea agglomerans	15 Strains isolated from rosaceous plants	UdG
P. agglomerans	850	CECT
Pseudomonas corrugata	124T	CECT
Pseudomonas fluorescens	EPS62e, EPS62e Nal	UdG
P. fluorescens	157 Strains isolated from rosaceous plants	UdG
P. fluorescens	Q2-87	USDA
P. fluorescens	CHAO	IPS
P. fluorescens	JBR1-70	WAU
Pseudomonas putida	324T, 385, 845, 4064, 4518, 4584, 4633	CECT
Pseudomonas syringae pv *syringae*	45 Strains isolated from rosaceous plants	UdG
P. syringae pv *tomato*	DC3000	[49]
P. syringae pv *phaseolicola*	3635-95	UPN
Ralstonia solanacearum	125	CECT
Shewanella baltica	323T	CECT

[a] UdG, Universitat de Girona (Spain); USDA, United States Department of Agriculture-Agricultural Research Service (USA); IPS, Institute of Plant Sciences (Switzerland); WAU, Wageningen Agricultural University (The Netherlands); UPN, Universidad Pública de Navarra (Spain); CECT, Colección Española de Cultivos Tipo (Spain).

obtained from a commercial orchard in Girona (Spain), while the self-rooted pear plants (cultivar Conference) were obtained by micropropagation (Agromillora Catalana, S.A., Barcelona, Spain). Fruits were surface-disinfected by immersion for 1 min in a diluted solution of sodium hypochlorite (1% active chlorine), washed twice by immersion in distilled water, and left to air dry. Each fruit was perforated four times with a tip (≈5 mm deep) before biological control agent treatment and pathogen inoculation. Individual pear flowers were obtained from detached pear branches with flower buds that were taken at the dormant bud development stage. They were then forced to bloom in an environmental chamber [34]. Open blossoms were detached from the branches and flowers were individually maintained with the cut peduncle submerged in 1 ml of 10% sucrose solution in 1.5 ml tubes placed in plastic tube racks [35]. Plants were 2–3 years old, grown in 20-cm-diameter plastic pots. These plants were left outside the greenhouse during winter for chilling. During early spring, the plants were pruned to leave 3 or 4 shoots and were then forced to bud in the greenhouse. The plants were used when the shoots were about 3 or 4 cm length and had 5 or 6 young leaves. Before biological control agent treatment and pathogen inoculation, the three youngest expanded leaves of each shoot were wounded by a double incision (≈1 mm) perpendicular to the midrib, approximately in the middle of the leaf.

For efficacy assays, trials were performed for each type of material in which EPS62e was applied by depositing 10 μl of a cell suspension into the wounds produced in the immature fruits and young leaves or in the surface of the hypanthium in flowers. In detached flowers and immature fruits, the doses were 10^7 and 10^8 CFU ml^{-1} and a trial was carried out. For the whole plants two independent trials were undertaken, the first trial at 10^7 and the second at 10^8 CFU ml^{-1}. Treated plant material was covered with plastic bags and incubated for 24 h at 21 °C, high relative humidity, 16 h of fluorescent light and 8 h dark. It was then inoculated with 10 μl of *E. amylovora* EPS101 suspension at 10^7 CFU ml^{-1}. Plant material was covered again with plastic bags, and incubated under the same conditions. The experimental design for each treatment consisted of three repetitions of nine immature fruits, eight flowers or three plants. Non-treated controls with water or inoculated with the pathogen were included.

Disease was evaluated as incidence of infection for immature fruits or flowers after 8 days of pathogen inoculation, and as severity for potted plants after 10 days of pathogen inoculation. Severity was determined for each leaf on a scale from 0 to 3: 0, no symptoms; 1, necrosis around midrib; 2, total midrib necrosis and 3, necrosis progression through petiole. ANOVA was performed to test the effect of the biocontrol application on the inhibition of *E. amylovora* infections. The means were separated according to the Tukey test ($P \leqslant 0.05$).

2.3. RAPD and U-PCR analysis

Several primers were tested in order to obtain specific amplified products for the strain EPS62e compared to eight other *P. fluorescens* strains (EPS263, EPS270, EPS288, EPS375, EPS817, Q2-87, CHAO and JBR 1-70). The primers were used alone following a RAPD procedure and as pairs according to a U-PCR method (Table 2). For RAPD and U-PCR assays, genomic DNA from strains was extracted following the methodology described by Keel et al. [36] with minor modifications. One isolated colony was grown in 600 μl of LB

Table 2
RAPD and U-PCR primers used

Primer type	Primer	Sequence 5′ → 3′	Reference
RAPD	PC1	GCG CAG ATC TAG CGC CTC GCC GCC GAA	[37]
	GAC	CCG TTA TTG CGC CCG	[36]
	M12	GGG ACG TTG G	[36]
	BOX	CTA CGG CAA GGC GAC GCT GAC G	[50]
U-PCR	PCA1	CCG CGT TGT TCC TCG TTC AT	[51]
	PCA2	TTG CCA AGC CTC GCT CCA AC	[51]
	PHL1	GAG GAC GTC GAA GAC CAC CA	[51]
	PHL2	ACC GCA GCA TCG TGT ATG AG	[51]
	PIR1	TCA AGG ACA AGC CGA CCG AGT	This work
	PIR2	GCA GCC CGA ACA GCA CGA AGT	This work
	PLT1	CGG AGC ATG GAC CCC CAG C	[50]
	PLT2	GTG CCC GAT ATT GGT CTT GAC CGA G	[50]

broth at 25 °C for 24 h. Then, 10 μl from the culture was transferred to 90 μl of lisis buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% p/v Tween 20) and incubated at 99 °C for 10 min. The extracted DNA was stored at −20 °C for later use. Amplification reactions were performed in a final volume of 25 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM of primer, 1.5 U of *Taq* DNA polymerase (Invitrogen, CA, USA) and 5 μl of the extracted DNA. RAPD and U-PCR fingerprints were obtained using the thermal cycle conditions described by Moënne-Loccoz et al. [37] with minor modifications (2 cycles at 94 °C for 5 min, 40 °C for 5 min and 72 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min). The amplified products were visualized by gel electrophoresis in a 1% agarose gel with 1× TAE, stained with ethidium bromide.

2.4. Cloning and sequencing selected RAPD and U-PCR fragments

Only PCA1, PHL1 and PHL2 primers were retained for their ability to amplify three discriminatory fragments of EPS62e DNA: the RAPD fragment of 450 bp obtained with PCA1, and the two U-PCR fragments of 900 and 1000 bp obtained by combining PHL1 and PHL2. These three fragments were selected, excised from agarose gels and cleaned according to the manufacturer's instructions with a QIAEX II kit (QIAGEN GMBH, Hilden, Germany). The purified fragments were cloned using TOPO TA Cloning® Kit for sequencing (Invitrogen). The cloned fragments were amplified using M13F and M13R primers and separated by gel electrophoresis to verify the correct length of the inserts prior to sequencing. All fragments were sequenced in both directions using BigDye Terminator v3.0 Ready Reaction cycle sequencing kit (PE Applied Biosystems) and ABI Prism™ 310 sequencer. For the 900 and 1000 bp fragments, besides the use of M13F/R, internal primers were designed using Primer Express™ software (PE Applied Biosystems, MA, USA). The sequences obtained were analysed for potential homologies using BLAST search to screen GenBank database.

2.5. Design of SCAR primers and PCR conditions

One SCAR primer pair was designed for each RAPD or U-PCR fragment using Primer Express™ software (PE Applied Biosystems), targeting a shorter internal region without homologies against known sequences from the database. SCAR primers were named after the original length from the RAPD or U-PCR fragment: SCAR 450F (GGCGCGCAACTGCTTT) SCAR 450R (CGGTTAGATCCGACAAGATTAGAG), SCAR 900F (CTCGCGTTGAGAGCAGAGAAC), SCAR 900R (TGGGACTATCGCTCACCATTTG), SCAR 1000F (CCTCGAACTCGTGGTTATGGT) and SCAR 1000R (CCAGGACTTTATACATCTGCAGCCTT), and they amplified 177, 392 and 378 bp fragments, respectively. The amplification reaction was performed in a final volume of 50 μl containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 0.75 U of *Taq* DNA polymerase (Invitrogen) and 5 μl of the extracted DNA. The thermocycle conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, elongation at 72 °C for 30 s and ending with an elongation step at 72 °C for 7 min.

2.6. Specificity and sensitivity of SCAR primers

Primers specificity was assessed against pure cultures of known bacterial strains and field samples. DNA from 162 strains of *P. fluorescens* and 75 strains of closely related species (*Pseudomonas syringae*, *Pseudomonas putida*, *Pseudomonas corrugata*, *Ralstonia solanacearum*, *Shewanella baltica*, *P. agglomerans* and *E. amylovora*) was tested with the three SCAR primer pairs. A total

of 61 field samples from plants were collected between March and June 2002 from Blanquilla and Williams pear trees from orchards in Zaragoza (Spain), where EPS62e had never been applied before. Samples containing from 5 to 7 g of leaves were homogenized (Masticator, IUL Instruments, England) with 50 ml of extraction buffer (0.14 M NaCl, 0.26 M $NaH_2PO_4 \cdot 2H_2O$, 0.75 mM $Na_2HPO_4 \cdot 12H_2O$, 2% PVP-10, 1% Manitol, 10 mM ascorbic acid, 10 mM L-glutathione reduced) for 60 s. DNA was extracted using the Llop et al. [38] procedure. Plant homogenate containing the microbiota extracted was centrifuged at 10,000*g* for 10 min and the pellet was again suspended in 500 μl of extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP). After 1 h of shaking, tubes were centrifuged at 5000*g* for 5 min and 450 μl was transferred to a new tube where 450 μl of isopropanol was added. Precipitation took place over at least 1 h, and after centrifugation at 13,000*g* for 10 min, the DNA was dried and suspended again in 200 μl of sterile ultra pure water. This DNA from field samples containing the microbiota and plant material from pear trees was tested in PCR with the three SCAR primer pairs. A negative control without DNA and a positive control with EPS62e DNA were included in each PCR run.

The sensitivity of the three SCAR primer pairs was tested by PCR. A cell suspension of known EPS62e concentration was serially diluted (1:10) in 1.5 ml tubes prefilled with 900 μl of plant extracts. The extracts were composed of 5 g of pear leaves in 45 ml of extraction buffer and homogenized for 90 s. Each suspension followed the DNA extraction procedure described by Llop et al. [38] and 5 μl was used in PCR as described above. Twenty microlitres of the amplified product was visualized by gel electrophoresis in a 2% agarose gel with 1× TAE and stained with ethidium bromide.

2.7. *Monitoring* P. fluorescens *EPS62e on pear leaves by Plating-PCR and MPN-PCR methods*

A set of 21 self-rooted pear plants (Conference cultivar), aged between 2–3 years and of 40–50 cm in height, were grown under greenhouse conditions. Two independent experiments were then carried out. Eighteen plants were sprayed with a water suspension containing 10^8 CFU ml^{-1} of EPS62e Nal in the first experiment and 5×10^8 CFU ml^{-1} in the second. The three remaining plants were sprayed with sterile water as a non-treated control. Plants were sampled for almost a month with three plants being collected at each sampling. Each sample was composed of 9 leaves from a single plant that were weighed and homogenized in 30 ml of extraction buffer for 60 s. For EPS62e quantification, samples were evaluated by two techniques: Plating-PCR and MPN-PCR. For the Plating-PCR method, 50 μl of serial 10-fold dilutions of each sample were dropped on an LB agar medium containing 50 μg ml^{-1} of nalidixic acid (three replicates per dilution) [39]. The plates were incubated at 25 °C for 24 h and the colonies were subsequently counted. Several representative colonies were randomly picked for PCR analysis with SCAR primers. For the MPN-PCR method, 96-well microtiter plates were pre-filled with 180 μl per well of LB broth containing 50 μg ml^{-1} of nalidixic acid, and 20 μl of each sample were transferred into the first row (3 wells per sample) and the following rows were serially diluted (1:10). Microtiter plates were incubated at 25 °C for 24 h and the last three dilutions with positive growth were analysed by PCR with SCAR primers. EPS62e Nal population level was estimated by using MPN tables with its standard error and a 95% confidence interval. Results from both methods were expressed as CFU $(g\ f.w.)^{-1}$.

3. Results

3.1. *Efficacy assays*

Pseudomonas fluorescens EPS62e significantly reduced not only the incidence of infections caused by *E. amylovora* in immature fruits and flowers but also the severity in pear plants at both doses tested (Fig. 1). The incidence in non-treated controls in flowers and immature fruits was 100%, while the severity on plants was between 94% and 79% for the trials 1 and 2, respectively. The preventive treatment with EPS62e decreased the incidence from 100% to 67% in flowers, from 100% to 53% in immature fruits and the severity from 94% to 27% in potted plants when it was applied at 10^7 CFU ml^{-1}. When applied at 10^8 CFU ml^{-1}, values decreased to 42%, 8%, and 2%, respectively.

3.2. *RAPD and U-PCR analysis*

Among the 12 primers tested, only PCA1 and PHL1/PHL2 gave rise to three reliable and reproducible polymorphic fragments which differentiated the strain EPS62e from other *P. fluorescens*. One RAPD fragment of 450 bp obtained with PCA1 (Fig. 2(a)) and two U-PCR fragments of 900 and 1000 bp obtained with PHL1/PHL2 primer pair (Fig. 2(b)) were selected. The sequences of the three selected fragments were analysed by BLASTA software and no significant homologies to the known sequences in the database were found.

3.3. *Development of SCAR markers*

The RAPD and U-PCR sequences of 450, 900 and 1000 bp were analysed by Primer Express™ software in order to design an internal primer pair for each that would amplify a shorter fragment. The annealing

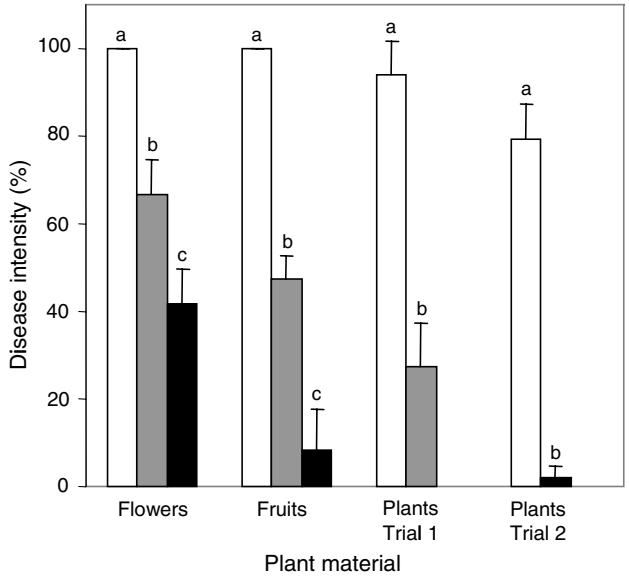

Fig. 1. Disease intensity in detached flowers, immature fruits, and potted pear plants treated with EPS62e at 10^7 (▨), 10^8 CFU ml^{-1} (■) or non-treated (□) and subsequently inoculated with *E. amylovora* EPS101. Confidence intervals for the mean are shown over the bars. Different letters within the same plant material and trial indicate significant differences between means according to Tukey's test ($P \leqslant 0.05$).

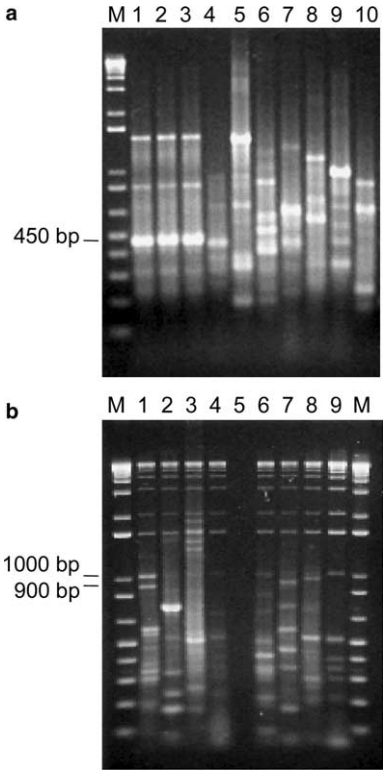

Fig. 2. RAPD and U-PCR profiles of several strains of *P. fluorescens*. (a) RAPD with PCA1 primer for (1–2) EPS62e, (3) EPS62e Nal, (4) Q2-87, (5) CHAO, (6) JBR1-70, (7) EPS817, (8) EPS263, (9) EPS375, (10) EPS288. (b) U-PCR with PHL1/PHL2 primer pair for (1) EPS62e, (2) Q2-87, (3) CHAO, (4) EPS270, (5) negative control (without DNA), (6) EPS817, (7) EPS263, (8) EPS375, (9) EPS288. (M) 1Kb Plus DNA Ladder (Invitrogen). The three amplified fragments (450, 900 and 1000 bp) suitable for developing SCAR primers are indicated.

temperature was optimized at 65 °C to obtain stringency PCR conditions, minimising the possibility of non-specific hybridizations with non-target DNA. The SCAR primers were named after the RAPD or U-PCR fragments length: SCAR 450, SCAR 900 and SCAR 1000. Each primer pair showed a single amplification product with the expected length of 177, 392 and 378 bp (Fig. 3).

3.4. Specificity and sensitivity of SCAR primers

The specificity of the SCAR primers designed was tested against a large number of strains and field samples: 161 strains of *P. fluorescens*, 75 strains of related species and 61 field samples of plants which represented a large diversity of natural microbial communities from the pear tree phyllosphere. The SCAR 450 and SCAR 900 primers were specific for the amplification of genomic DNA from EPS62e and EPS62e Nal, without showing any unspecific amplification in other strains and the microbiota of field samples tested (Fig. 3(a) and (b)). Nevertheless, even though neither the other species nor the field samples were amplified, the SCAR 1000 primer pair amplified a fragment of the same size in 9

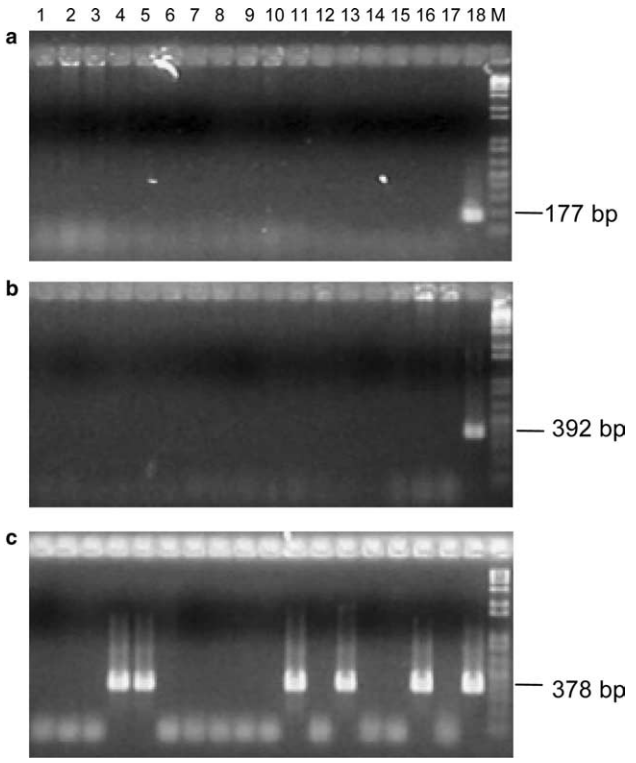

Fig. 3. Screening of the SCAR 450 (a), 900 (b) and 1000 (c) primer specificity among a group of *P. fluorescens* strains. (1) EPS581, (2) EPS582, (3) EPS583, (3) EPS584, (4) EPS585, (5) EPS586, (6) EPS587, (7) EPS588, (8) EPS589, (9) EPS590, (10) EPS591, (11) EPS597, (12) EPS598, (13) EPS599, (14) EPS600, (15) EPS601, (16) EPS602, (17) negative control (without DNA), (18) Pf EPS62e. (M) 1Kb Plus DNA Ladder (Invitrogen).

of the 161 *P. fluorescens* strains tested (Fig. 3(c)). The correspondence of the 9 amplified products to the SCAR fragment of EPS62e was evaluated by enzyme restriction analysis and all amplification products followed the same restriction profile as the SCAR 1000 fragment (data not shown).

The sensitivity was then evaluated by the PCR in dilutions of EPS62e in plant extracts ranging from 10^7 to 10^2 CFU/PCR (Fig. 4). The SCAR 450 and SCAR 900 primer pairs detection limit was 10^2 CFU/PCR, whereas the SCAR 1000 primer pair was ten times less sensitive, i.e. 10^3 CFU/PCR. The SCAR 900 seemed to slightly increase the sensitivity compared with the SCAR 450 because of the higher intensity of the amplification signal at the limit of detection.

3.5. *Monitoring* P. fluorescens *EPS62e on pear leaves*

The population level of EPS62e was evaluated after its application on pear plants by spraying at 10^8 CFU ml^{-1} in the first experiment and 5×10^8 CFU ml^{-1} in the second experiment and using Plating-PCR and MPN-PCR. Fig. 5 shows the EPS62e population dynamics on pear leaves. During the first 17 days after treatment the strain levels gradually decreased by two orders of magnitude (from 7 to 5 log CFU (g f.w.)$^{-1}$), then remaining stable at 4–5 log CFU (g f.w.)$^{-1}$ for the next 11 days. We did not observe any remarkable differences between either the two independent assays performed or between the two monitoring techniques. All the colonies tested by PCR in the plating method were identified as EPS62e. We found a linear relationship between the results obtained by the two techniques ($y = 0.997 \times -0.426$; $R^2 = 0.978$; $P < 0.001$). Both techniques developed for monitoring EPS62e achieved a detection level around 3 log CFU (g f.w.)$^{-1}$.

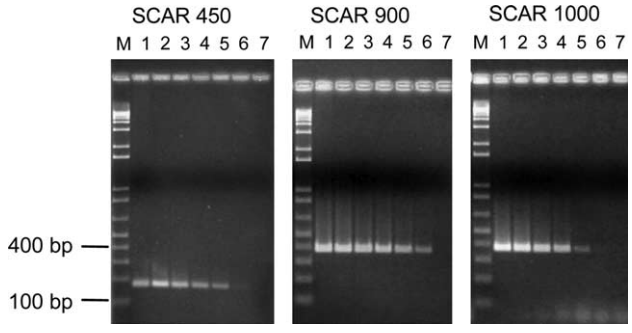

Fig. 4. Amplified fragments from *P. fluorescens* EPS62e with SCAR 450, 900 and 1000 primer pairs. The EPS62e cell suspension was diluted in plant extracts at a final concentration of (1) 10^7, (2) 10^6, (3) 10^5, (4) 10^4, (5) 10^3 and (6) 10^2 CFU/PCR, (7) negative control (without DNA), (M) 1Kb Plus DNA Ladder (Invitrogen).

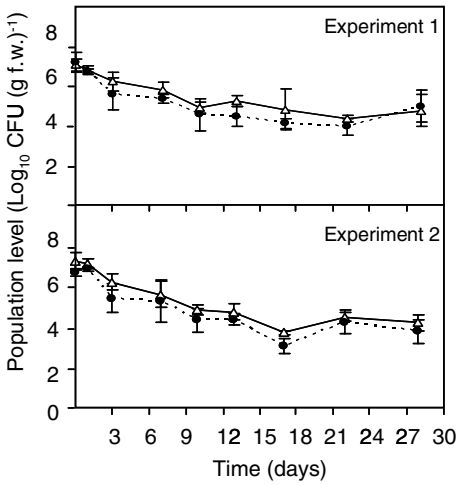

Fig. 5. Population dynamics of *P. fluorescens* EPS62e on pear leaves under greenhouse conditions after application at 10^8 (Experiment 1) or 5×10^8 CFU ml^{-1} (Experiment 2). Values were determined by the plating-PCR method (continuous line) and the MPN-PCR method (dashed line). Each point is the mean of three plants. The confidence interval is represented by a vertical bar.

4. Discussion

Pseudomonas fluorescens EPS62e isolated from pear fruit surface is a potential biological control agent against fire blight because it significantly reduced *E. amylovora* infections on susceptible organs and whole pear plants when applied in preventive treatments under greenhouse conditions. Ongoing studies to develop this strain as a biopesticide for fire blight control have brought out the need to have available a monitoring method for tracking EPS62e after field application.

Monitoring methods can be grouped into cultivation based methods (semi-selective or selective growth media) or nucleic acid based methods (e.g., PCR technique) [12]. In cultivation based methods, antibiotics are generally used to allow the specific growth of the target strain, and the population level is estimated by plating and colony-forming units counting [40–42] or by the most probable number (MPN) procedure [43]. These microbiological methods have the advantage that only viable microorganisms are detected, but they would have a lack of specificity if there were non-targeted microorganisms with the same resistance marker in the environment [16]. Nucleic acid based methods have higher specificity but the lack of discrimination between living cells or free DNA would result in an overestimation of viable population levels. In the present work, we have combined the advantages of both types of analysis by means of attaching microbiological methods to a PCR-based detection.

SCAR markers have been commonly used for developing monitoring methods for biological control agents because they are natural sequences present in the genome which allow a simple specific detection by

PCR procedure. The most important and significant advantage of SCAR markers is that they do not require any prior knowledge of the strain genome. In the present study, we have used RAPD and U-PCR techniques to find differential amplified fragments for the SCAR primer design. The RAPD has been a current technique to detect genomic polymorphisms since its development in 1990 [19,20]. Nevertheless, we have shown that the U-PCR is another useful technique for fingerprinting. It is generally accepted that the more genetically heterogeneous the species, the easier it is to find strain specific SCAR markers. One RAPD and two U-PCR sequences specifically tagged EPS62e. The fragments chosen were no longer than 1000 bp, thus, simplifying the later cloning and sequencing steps. Even though the RAPD or U-PCR profiles could become monitoring methods themselves, it is preferable to convert them into SCAR markers because they avoid the need to culture the strain before analysis, are more specific as they target a known sequence, are less sensitive due to stringency of the PCR conditions and they simplify the detection with a single band instead of a profile. Therefore, the three sequences were used to design internal SCAR primer pairs, SCAR 450, SCAR 900 and SCAR 1000, minimising the length of the amplified product.

The specificity of SCAR 450 and SCAR 900 was confirmed by the absence of amplification signal in all strains and field samples tested, whereas SCAR 1000 was semi-specific because it amplified some of the *P. fluorescens* strains apart from EPS62e. The lack of specificity of some of the previously designed SCAR primer pairs has also been observed when developing SCAR markers for the biological control agent *Beauveria bassiana* GHA [44]. The fact that we have obtained two specific SCAR markers valuable in EPS62e detection is important in case of DNA rearrangements or point mutations in the strain, which would cause the loss of the target sequence. This source of variability has been reported in related species such as *Pseudomonas stutzeri* [45].

Sensitivity was evaluated by mimicking real conditions of field sampling by mixing several dilutions of EPS62e strain with plant extracts where exogenous DNA and PCR inhibitors would be present. We adapted the extraction procedure used for the detection of *E. amylovora* [46]. This will save laboratory work needed for simultaneous detection of either the pathogen or the EPS62e using the appropriate primer pair in PCR. The best sensitivity was achieved by SCAR 450 and SCAR 900, with a threshold level of 10^2 CFU/PCR. Taking into account the standard conditions of sample processing, DNA extraction and PCR sensitivity, the detection level is around 10^4 CFU/blossom or unit organ. This threshold is low enough with regard to population levels required to obtain an effective biocontrol on blossoms where a population size of 10^5–10^6 CFU/blossom of the antagonist strain *P. fluorescens* A506 is needed before the pathogen arrival [47]. However, the sensitivity could be improved by developing a nested PCR within the SCAR fragment [29] or by labelling primers for fluorogenic assays [31].

To study the population dynamics of EPS62e we have compared two microbiological techniques attached to PCR: the plating method with randomly picked colonies tested by the PCR and the MPN-PCR method. The plating procedure involved time consuming CFU counting and only a few colonies were randomly chosen for being verified by the PCR. In contrast, the MPN-PCR procedure used 96-well microtiter plates which improve routine analysis, made the quantification less liable to human errors (automated optical density reader), and all the dilutions used for quantification were verified by the PCR. In order to improve the plating method a spiral plating device could be used, which saves time by eliminating dilutions required. Moreover, it could be partnered with an automated counting system to reduce the time and errors of manual counting, even though this equipment would increase the cost of the method. Nevertheless, the main advantage of the MPN-PCR method over plating is that the first could be used without the amendment of nalidixic acid as all the microwells showing growth are taken for PCR evaluation, whereas in plating (without supplemented antibiotic) it is almost impossible to test all the colonies by PCR. This advantage is remarkable when developing a new biopesticide as the wild type strain instead of the antibiotic resistant mutant is preferred for registration and use. We found a linear relationship between results obtained by the two techniques, with a slope close to 1. Thus, both methods were useful for the quantification of EPS62e population level. Moreover, differences in sensitivity between plating and MPN-PCR were not observed for all the concentrations tested and the detection level obtained was around 3 log CFU $(\text{g f.w.})^{-1}$ of leaves. However, our results contrasted with the report of Landa et al. [48] who studied phloroglucinol producing strains of *P. fluorescens* in roots where MPN-PCR was almost 10 times more sensitive (detection level of 3.26 log CFU $(\text{g f.w.})^{-1}$) than the plating method (detection level of 4.0 log CFU $(\text{g f.w.})^{-1}$). Despite this, the limit of detection obtained in our study for the MPN-PCR targeted to strain EPS62e was of the same order.

The strain EPS62e colonized and survived well for almost a month on the leaves of pear plants under greenhouse conditions. The population levels remained stable at 4–5 log CFU $(\text{g f.w.})^{-1}$, indicating that EPS62e has the potential to colonize plants exposed to fluctuations of temperature and relative humidity, under limited nutrient availability, as has been described for other bacteria in the phyllosphere [13]. The population level achieved by EPS62e is in accordance with the fact that

colonization ability is one of the main factors involved in *E. amylovora* biocontrol [33].

In summary, we have developed two specific SCAR markers that allow the unambiguous detection and quantification of EPS62e after field application by coupling plating and MPN methods to PCR. The monitoring tools developed have evaluated EPS62e behaviour on pear plants under greenhouse conditions, which bore out the capacity of the strain for colonization. Studies are ongoing to develop a real time PCR with fluorescent probes targeted in the SCAR sequence. This method will allow further field studies of biocontrol efficacy, colonization and survival of the strain in orchards or nurseries where fire blight is present.

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