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Monitoring system for the biocontrol agent *Pichia anomala* strain K using quantitative competitive PCR-ELOSA

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A quantitative-competitive PCR (QC-PCR) method with enzyme-linked oligosorbent assay (ELOSA) was developed to monitor *Pichia anomala* strain K, a biocontrol agent against postharvest diseases on apples. The procedure involved: (i) extraction of strain K DNA; (ii) coamplification of a constant amount of the extracted DNA (containing a strain K DNA marker of 262 base pairs (bp) specifically amplified with SCAR primers K1 and K2) with an internal standard (IS) titration; and (iii) differential hybridization with two specific biotinylated probes either for the target or for the IS sequence on microplate. The IS sequence differed from the target by only a short internal region of 35 bp providing the differential detection between both sequences. Both target and IS sequences proved to be competitive in PCR as well as in sandwich hybridization. Two copies of the target sequence were detected in the strain K genome by means of enzymatic restriction and Southern blot analysis. Varying amounts of strain K cell suspension were quantified in the phosphate buffer used for recovering cells from the apple surface. An accurate estimate of the strain K population was obtained from 10^3 to 10^6 yeast cells per apple. The threshold of the method was found to be at 1000 colony-forming units per apple, which was around 100 times more sensitive than the plating method for monitoring strain K.

Keywords: apple, biological control, *Botrytis cinerea*, enzyme-linked oligosorbent assay, *Penicillium expansum*, SCAR

Introduction

Pichia anomala strain K, an epiphytic yeast isolated from apple, was previously selected for its high and reliable protective activity against *Botrytis cinerea* and *Penicillium expansum*, two of the most devastating pathogens on harvested apples (Bondoux, 1992; Jijakli *et al*., 1993). The monitoring of strain K requires the development of a specific method able to quantify the population of the biocontrol agent (BCA) and to distinguish it from the indigenous microflora. As the protective effect of strain K is closely related to colonization (Jijakli & Lepoivre, 1998), evaluating the ecological fitness of *P. anomala* strain K after treatment on apples is critical for the interpretation and prediction of its biocontrol efficacy in relation to several parameters (methods of BCA application, environmental conditions of storage rooms, integration of BCA to other sanitary measures). Finally, this method will be also used to fulfil some eco-toxicological requirements for the registration procedure.

In comparison with the classical plating technique, polymerase chain reaction (PCR) methods offer reliable approaches for qualitative and quantitative monitoring of a specific microbial strain in the environment. It is also an alternative tracing technique in case of limitations of microbial cultivability on growth media. In this respect, quantitative-competitive PCR (QC-PCR) is widely used to quantify bacterial pathogens and phytoplasmas (Hu *et al*., 1997; Berges *et al*., 2000).

In a QC-PCR assay, target DNA is coamplified with an internal standard (IS) DNA. Quantification is achieved by comparing the relative yield of the PCR products amplified from the target and from the IS that should remain constant during the amplification. For this reason, careful design of the IS DNA must ensure that it is amplified with the same efficiency as the target sequence.

The IS and the target share primer sites and differ in size or in internal sequence. For example, Alexandre *et al*. (1998) reported a method whereby target and IS sequences were amplified with the same primers and were identical except for a very short region. Target and IS DNA were differentiated using enzyme-linked oligosorbent assay (ELOSA) (Zammatteo *et al*., 1995). The ELOSA method involves the binding of both target and IS sequences in the same microwell using a single capture probe followed by differential binding of specific detection probes for each amplification product. The combination of PCR and ELOSA is highly specific, based on primers and DNA probe hybridization specificity.

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The identification of a specific strain K natural DNA marker was previously obtained using RAPD (random amplified polymorphic DNA) analysis (De Clercq *et al*., 2003). SCAR (sequence-characterized amplified region) primers (K1 and K2) were designed and proved to be specific for amplification of a 262-bp *P. anomala* strain K SCAR marker (De Clercq *et al*., 2003).

The aim of this work was to develop a tracking method for the strain K using a QC-PCR-ELOSA with a SCAR marker as target.

Materials and methods

Construction of the internal standard DNA sequence

The IS or competitor DNA sequence was constructed through the principle of splice overlap extension (SOE) as described by Zimmermann & Mannhalter (1996).

The 3′ extremity of primers KIS1 (5′TAAgTCCTAggTggAATTATggAgcTTTTTATggAgAAcccgcAT3′) and KIS2 (5′TccAccTAggAcTTAcTAcAAcTAgTgTcAgATATTTTATcAgTg3′) was composed of a 20-bp sequence homologous to an internal sequence of the target (strain K SCAR marker). KIS1 and KIS2, when combined with their respective associated SCAR primers K2 (5′ccTggcccTAATTcTTcTcTTgTc3′) and K1 (5′AggAAgAAggATggAAAgAATgAgg3′), enabled PCR amplification of two distinct fragments with sizes of 187 and 90 bp, respectively (Fig. 1). PCR reactions were performed in a final volume of 50 μL containing 1 × PCR buffer (Roche, Basel, Switzerland), 0·2 μM dNTP, 0·2 μM of each primer, 1 U of *Taq* DNA polymerase (Roche) and 10 ng of purified plasmid DNA (PCR 2·1) containing the target sequence and previously obtained by following the TA cloning kit instructions (Invitrogen, Merelbeke, 9820, Belgium). PCR amplification conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, finishing with a single elongation step at 72°C for 5 min. Primers KIS1 and KIS2 also had a tail sequence of 25 bp at 5′ that was not homologous to the target sequence and that enabled dimerization of the two PCR products based on the 15 homologous bases at the 5′ extremity. The dimerization conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 15 cycles at 95°C for 1 min, 36°C for 1 min and 72°C for 1 min, finishing with an elongation step at 72°C for 5 min. PCR amplification was then performed with the external primers K1 and K2 on the dimerized sequences (1 × PCR buffer (Roche), 0·1 μM dNTP, 0·4 μM of both primers (K1 and K2) and 1 U of *Taq* DNA polymerase (Roche) in a final volume of 50 μL. The generated IS DNA sequence was cloned into the plasmid PCR 2·1 using the TA cloning kit (Invitrogen) and sequenced (T7 sequencing kit; Amersham Bioscience, Uppsala, Sweden). The IS sequence contained the same percentage of G/C as the target, and only differed from the target by a short internal sequence of 35 bp (Fig. 1).

Target and internal standard amplification conditions

The amplification reactions with primers K1 and K2 were performed in a final volume of 50 μL containing 1 × PCR buffer (Roche), 0·2 μM dNTP, 0·2 μM of each primer, 1 U of *Taq* DNA polymerase (Roche) and 10 ng of plasmid harbouring the target, the IS or purified strain K genomic DNA (Ausubel *et al*., 1987). The thermocycle conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, finishing with an elongation step at 72°C for 5 min.

ELOSA assay

The ELOSA design (Fig. 2) was based on the strain K-specific SCAR sequence of 262 bp. The capture probe common to both target and IS sequences was 160 nucleotides long. The target detection probe (5′cgcATTcgcTcAAAccATgATcAATgATTTcccAA3′) and the IS detection probe (5′gcTccATAATTCCACCTAggACTTAcTAcAAcTAg3′) were the same size (35 nucleotides) and were separated from the common capture probe by a gap of two nucleotides. The capture probe was produced by PCR amplification with the primers KTRAP1 (5′P-ccTggcccTAATTcTTcTcTTgTc3′) and KTRAP2

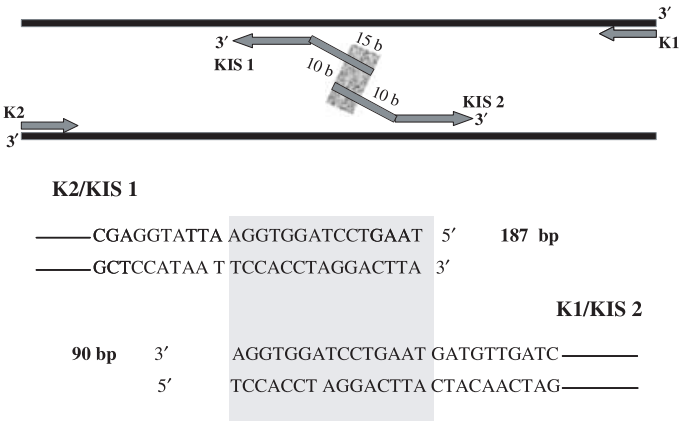

Figure 1 Design of internal standard DNA sequence using the splice overlap extension (SOE) method.

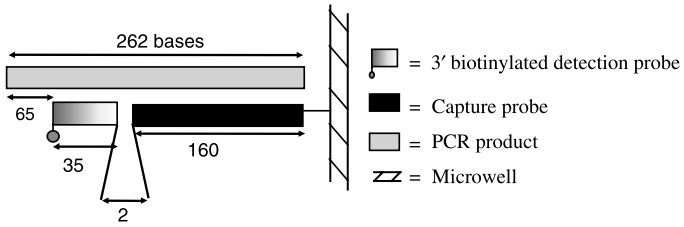

Figure 2 Schematic representation of the enzyme-linked oligosorbent assay (ELOSA) designed for the detection of the strain K-specific SCAR sequence (262 bp).

(5′TTTATggAgAAcccgcATTTTcgTT3′), both at a concentration of 0·4 μM in presence of 1 × PCR buffer (Roche), 0·2 μM dNTP, 1 U of *Taq* DNA polymerase (Roche) and 10 ng of plasmid target DNA. The PCR amplification conditions of the target and the IS sequences were applied, modified with the number of cycles to 45 and the annealing temperature fixed at 55°C. The capture probe was covalently fixed onto microwells (400 ng/well) following the protocol of Rasmussen *et al*. (1991).

Sandwich hybridization described by Chandelier *et al*. (2001) was optimized for the sequences (25 ng of detection probe and hybridization at 65°C for 2 h) and was performed on PCR products (10-fold diluted) of the target and the IS sequences.

Number of target sequences in the genome of strain K

Ten micrograms of purified genomic DNA, obtained using the method of Ausubel *et al*. (1987), were cut with *Bsm*I, *Mbo*I, *Hae*III or *Hpa*II according to instructions of MBI Fermentas Inc (Hanover, MD, US) and purified with ethanol precipitation. The restriction fragments were separated by gel electrophoresis, transferred to a nitrocellulose membrane and exposed for hybridization with a radioactive labelled target-P32 probe (Amersham Bioscience) previously amplified with K1/K2 primers.

Quantification of strain K cells

Strain K cells cultivated for 1 day on potato dextrose agar (PDA) medium at 25°C were harvested in sterile physiological water (0·85% w/v NaCl) and quantified by absorbency measurements of the suspension at 595 nm. Different amounts of yeast cells (8×10^3, 8×10^5, 8×10^6 and 3×10^7) were suspended in 250 mL of phosphate buffer (0·016 M K_2HPO_4, 0·034 M KH_2PO_4 and 0·05% Tween 80 at pH 6·5), which is used to recover yeast cells on treated apple surfaces. Cell pellets were obtained from the suspension by vacuum filtration with a 0·45 μM filter. The filter containing strain K cells was placed in a 15 mL Falcon tube with 2 mL of phosphate buffer. The tubes were twice vortexed for 20 min. The filter was removed and tubes were centrifuged at 1599 ***g*** for 15 min at room temperature. The pellet was suspended in 50 μL of Tris-HCl (40 mM, pH 8) and EDTA (5 mM). For DNA extraction, 100 μL of lysis buffer [NaOH 0·2 M and sodium dodecyl sulphate (SDS) 1%] was added and the tubes were incubated in a water bath at 100°C for 30 min. Cellular debris and SDS were precipitated with 75 μL of a solution containing 3 M potassium acetate and 6·9 M acetic acid incubated on ice for 10 min. After centrifugation (7 min at 15 700 ***g***), the supernatant was recovered and DNA was ethanol precipitated and suspended in 50 μL of distilled water.

During QC-PCR-ELOSA, several dilutions of the IS plasmid DNA were coamplified with 1 μL of the extracted genomic DNA with the primers K1/K2. For quantification, a linear relationship was plotted [the logarithm of the ratio of optical density (OD) values of the target and IS sequences against the logarithm of the initial concentration of the IS plasmid (DNA)]. A correction factor was obtained from the ratio of initial number of cells suspended in the phosphate buffer (estimated by absorbency measurements at 595 nm) and the QC-PCR-ELOSA experimental quantification.

Results

Validation of the QC-PCR-ELOSA method

The specificity of both detection probes was evaluated on target (strain K SCAR marker) and IS amplified products (Fig. 3a). The target detection probe hybridized to the immobilized target but not to the immobilized IS sequence. Cross-hybridization was not observed for the IS detection probe. In order to evaluate the competition between both sequences in ELOSA assay, hybridization of increasing equimolar mixtures of the target and the IS amplified products (0·1, 0·5, 1, 10 and 100 ng) was performed in the same microwell (Fig. 3b). Both products were detected with the same efficiency starting at 500 pg of amplification products.

To estimate the PCR amplification efficiency of target and IS sequences, increasing equimolar mixtures of the target and the IS purified plasmids (from 10^2, 10^3, 10^4 to 10^8 copies in five replicates) were amplified together in the same PCR reaction with K1 and K2 primers (Fig. 4). Detection and quantification of the endpoint PCR products were performed using the QC-PCR-ELOSA technique. Whatever the initial sequence (target or IS), the final amount of PCR products progressively increased when higher amounts than 10^3 initial copies were added (Fig. 4). After PCR, there was no significant difference in the amount of both sequences obtained (Snedecor's *F*-distribution, $P = 0{\cdot}289$) over the whole range of initial concentrations tested. The detection limit was estimated between 10^3 and 10^4 initial copy numbers, the threshold level being set at the double optical density value of the ELOSA negative control.

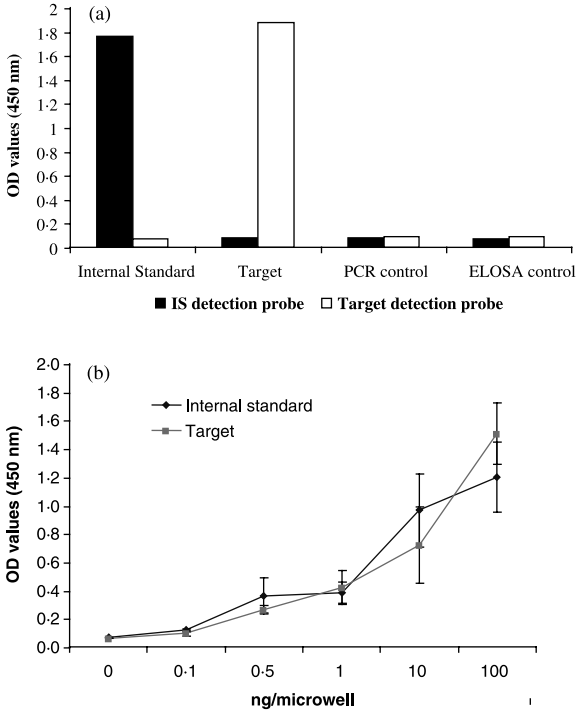

Figure 3 Hybridization specificity and amplification efficacy of the internal standard (IS) and target sequences in the quantitative-competitive PCR method with enzyme-linked oligosorbent assay (QC-PCR-ELOSA). (a) Optical density (OD) values after hybridization of the amplified products of the IS and the target sequences with IS or target detection probes in the ELOSA method. The PCR control and the ELOSA control were conducted without adding either DNA or amplified product, respectively. (b) OD values after co-hybridization in the same microwell of equimolar concentrations of amplified products of the IS and the target sequences. Each point is the mean of three independent hybridization reactions. The standard deviation of each mean is represented by a vertical bar.

Quantification of plasmid copy numbers with QC-PCR-ELOSA

A constant amount of the target (10^6 plasmid copy numbers) was coamplified with different and increasing amounts of IS plasmid DNA (ranging from 10^2 to 10^8 copies in three replicates per concentration) (Fig. 5). The endpoint amount of IS DNA increased strongly between 10^5 and 10^6 initial copies and reached a plateau between 10^7 and 10^8 copies (Fig. 5a). The target curve presented an opposite pattern: the highest amounts of the target after PCR were obtained in the presence of low amounts of the IS, and lower amounts of the target were produced in the presence of high copy numbers of the IS. The curves intersected around 10^6 plasmid copies. A linear relationship ($y = -0{\cdot}4702x + 2{\cdot}8541$; $r = 0{\cdot}97$) between the logarithm of the ratio of the OD signals from both sequences (y) and the logarithm of the initial copy number of the IS (x) was obtained (Fig. 5b). The initial copy number of the target ($1{\cdot}2 \times 10^6$) was deduced from the graph where endpoint amounts of both target and IS were identical (logarithm of the ratio of the OD values = 0).

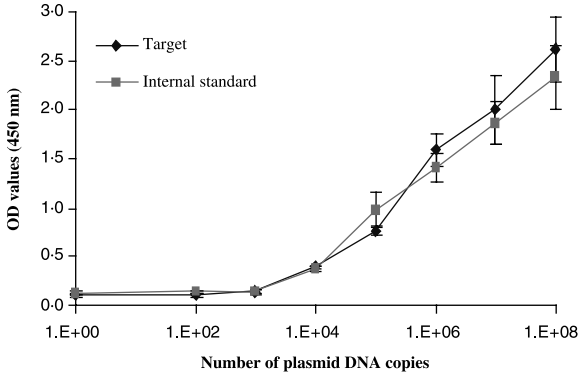

Figure 4 Relation between optical density (OD) absorbency and number of plasmid DNA copies of the target and the internal standard (IS) sequences after their coamplification and enzyme-linked oligosorbent assay (ELOSA) detection using initial equimolar mixtures of both purified plasmids (from 10^2, 10^3, 10^4 to 10^8 copies). Each point is the mean of five independent PCR amplifications. Standard deviation of each mean is represented by a vertical bar.

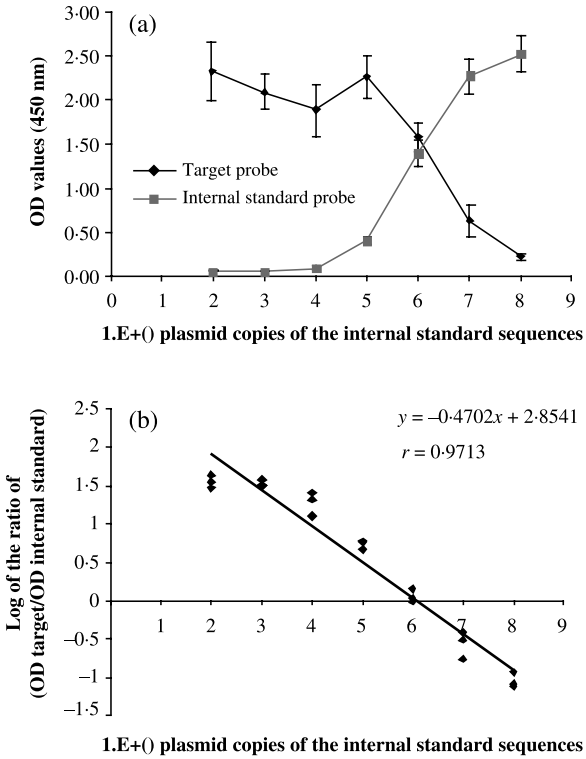

Figure 5 Detection and quantification of 10^6 target copy number by means of the quantitative-competitive PCR method with enzyme-linked oligosorbent assay (QC-PCR-ELOSA) in a competition plot with internal standard (IS) sequences where increasing amounts of IS purified plasmids (from 10^2 to 10^8) were coamplified with a constant amount of the target (10^6 copies). (a) Concentration curve of the target and IS sequences; each point is the mean of three independent PCR amplifications. The standard deviation of each mean is represented by a vertical bar. (b) Linear relationship obtained from the ratio of optical density (OD) target/OD IS hybridization signals plotted as a function of initial IS plasmid copy number.

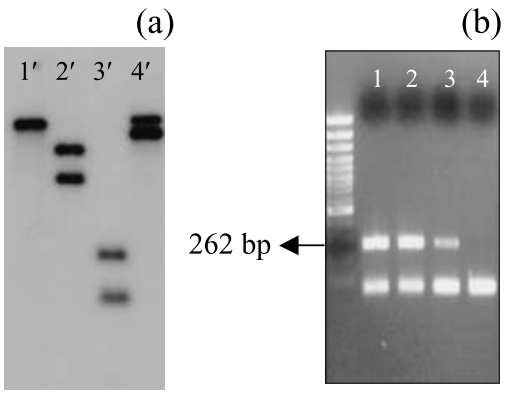

Figure 6 Determination of the copy number of the target fragment in the strain K genome. (a) Southern blot analyses of genomic strain K DNA after enzymatic restriction with *Hpa*II (1′), *Hae*III (2′), *Mbo*I (3′) and BsmI (4′) showing specific hybridization with a P32-labelled target probe. (b) PCR amplification of both fragments obtained after *Hae*III digestion (1 and 2), strain K genomic DNA (3), and negative control (4). Molecular weight marker (100-bp DNA ladder).

Determination of copy number of target in strain K genome

Enzymatic restriction with *Hpa*II and *Hae*III, which do not cleave the target sequence, was revealed after Southern blotting one and two fragments, respectively (Fig. 6a). Three fragments were expected with the use of *Mbo*I because this enzyme has two restriction sites in the target sequence, but the smallest fragment of 52 bases was not observed upon blotting. The use of *Bsm*I that cuts once within the target sequence resulted in two fragments. Restriction analysis with *Mbo*I, *Hpa*II and *Bsm*I indicates the presence of one single target sequence in the genome of strain K. However, the *Hae*III restriction profile indicates the presence of two target sequences in the genome. PCR amplification with the primers K1 and K2 was performed separately on both fragments obtained after target digestion with *Hae*III (Fig. 6b). Amplification was successful in both cases, revealing two copies of the sequence inside the genome of strain K.

Molecular monitoring of strain K

In order to evaluate the molecular monitoring method on strain K population, different amounts of yeast cells were suspended in a phosphate buffer and these samples were subsequently quantified by QC-PCR-ELOSA. This buffer has been shown to recover more than 99% of the cells from apple surfaces (De Clercq *et al*., 2003).

The quantification of the cell number by QC-PCR-ELOSA, the corresponding correction factor and the equations of the linear regression plots are presented in Table 1. The linear correlation coefficients of different cell concentrations ranged between 0·96 and 0·99. The correction factor between theoretical and experimental cell number estimations increased gradually with higher cell concentrations. For the lowest cell number, the QC-PCR-ELOSA quantification resulted in an overestimation (correction factor 0·25) as compared with theoretical quantification. This phenomenon gradually changed into an underestimation for increasing yeast cell amounts.

Discussion

Pichia anomala strain K is a reliable BCA for the protection of apples against postharvest diseases caused by *B. cinerea* and *P. expansum* and seems to act through its ability to colonize the apple surface, which explains the usefulness of tracking the population dynamics of the strain K after its application on apples.

The classical method for quantification of a particular BCA consists of counting colony-forming units (cfu) on a selective or semiselective solid medium (Mercier & Wilson, 1995; Leibniger *et al*., 1997; Lima *et al*., 1997, 1999; Usall *et al*., 2001; Johnson *et al*., 2000). This method has the advantage that only living microorganisms are detected.

Table 1 Quantification of different cell concentrations using the quantitative-competitive PCR method with enzyme-linked oligosorbent assay (QC-PCR-ELOSA)

Number of strain K cells[a]	QC-PCR-ELOSA quantification of strain K cell numbers[b]	Equation and correlation coefficient (r)	Correction factor (a/b)
$1{\cdot}6 \times 10^2$	$6{\cdot}3 \times 10^2$	$y = -0{\cdot}3468x + 1{\cdot}0744$ $r = 0{\cdot}97$	0·25
$1{\cdot}6 \times 10^4$	$3{\cdot}4 \times 10^4$	$y = -0{\cdot}5399x + 2{\cdot}6061$ $r = 0{\cdot}96$	0·5
$1{\cdot}6 \times 10^5$	$1{\cdot}5 \times 10^5$	$y = -0{\cdot}6183x + 3{\cdot}3889$ $r = 0{\cdot}98$	1·1
$0{\cdot}7 \times 10^6$	$0{\cdot}2 \times 10^6$	$y = -0{\cdot}5493x + 3{\cdot}1158$ $r = 0{\cdot}99$	3·5

[a]Number of strain K cells was obtained from optical density (OD) values at 595 nm of cells suspended in the phosphate buffer used to recover cells from the apple surface.
[b]Experimental quantification by QC-PCR-ELOSA of strain K cell number. The cell number was extrapolated from the target copy number (1 strain K cell = 2 target copy number). The target copy number was deduced from the relationship when endpoint amounts of both target and internal standard probes were identical ($y = 0$).

However, the unambiguous identification of a specific strain of a microorganism based on morphological characteristics remains difficult and limits the routine application of this approach. To overcome this lack of specificity, counting cfu on solid semiselective medium can be combined with PCR screening of randomly picked colonies. The combination of these techniques was used for the monitoring of other BCA, such as *Candida oleophila* (Chand-Goyal *et al*., 1999), *Trichoderma hamatum* (Abbasi *et al*., 1999) and *Metschnikowia* spp. (Schena *et al*., 2000). Plating followed by PCR was also successfully applied on *P. anomala* strain K (De Clercq *et al*., 2003). In that case, the identification of strain K was based on the PCR amplification of the SCAR marker of 262 bp (derived from a RAPD fragment) using primers K1 and K2. These SCAR primers were specific for strain K as confirmed by Southern blot analysis. However, only a percentage of the colonies growing on the semiselective medium were investigated by PCR and the number of cfu was subsequently estimated by extrapolation. Moreover, this combined method was time-consuming and therefore not easy to use routinely.

Therefore, QC-PCR-ELOSA based on this 262-bp SCAR marker was developed. For quantification, an IS was synthesized by SOE. The latter was also amplified by the primers K1 and K2, and had the same sequence as the target fragment, except for a sequence of 35 bp, which had been used to define ELOSA detection probes specific for either the target or the IS DNA. The optimized ELOSA detection system for the target and the IS sequences was capable of detecting 0·5 ng of amplified product, and was similar to the sensitivity level obtained by Alexandre *et al*. (1998) fixed between 0·5 and 5 ng.

In this study, it was demonstrated that both target DNA and IS DNA were amplified over eight orders of magnitude with similar efficiency, ratios of OD values obtained from both target and IS amplified products at equimolar initial concentrations being between 0·78 and 1·13 for all dilutions tested. This is in accordance with the work of Souazé *et al*. (1996), who demonstrated that a ratio between 0·66 and 1·5 ensured an accurate quantification.

In order to evaluate the quantification process, a known amount of target plasmid DNA (10^6 copies) was coamplified with different concentrations of IS plasmid DNA. The competition for dNTPs, enzyme and primers between target and IS is illustrated in Fig. 5(a). Since the relative amounts of both sequences are theoretically maintained along the amplification, a linear relationship was obtained when plotting their ratio as a function of the initial copy number of IS DNA (Fig. 5b), which enabled estimation of the number of target DNA sequences at $1{\cdot}2 \times 10^6$ copies, showing the accuracy of the quantification method.

The development of a molecular monitoring method using QC-PCR-ELOSA for the specific target of *P. anomala* strain K enabled quantification of the initial copy number of this fragment. However, the final objective of this study was to quantify the yeast cell number of strain K. To establish the relationship between the copy number of the target and the cell number, it was essential to determine the copy number of the target fragment present in the genome of strain K. Two copies of the target fragment were detected in strain K genome after *Hae*III digestion. Because of the diploid status of *P. anomala* strain K (Grevesse *et al*., 1998), two homologous sequences of the target fragment were detected in the strain K genome. A polymorphism on the two chromosomes in a *Hae*III restriction site beyond the target sequence could explain this result. Using the QC-PCR-ELOSA method, it was possible to quantify the number of yeast cells of *P. anomala* strain K suspended in the phosphate buffer which is used to recover the antagonist from the apple surface (De Clercq *et al*., 2003). Moreover, the yeast cell numbers that were tested represent a realistic range of strain K amounts expected on the surface of pre- or postharvest treated apples (Jijakli *et al*., 2002).

Quantification of strain K cells by QC-PCR-ELOSA has an acceptable level of accuracy within the range of 10^3–10^6 yeast cells per apple where high values of correlation coefficients were obtained. Beyond this range of strain K cells, the precision of the quantification method diminishes considerably. However, the sensitivity threshold of this method with respect to its accuracy is situated around 1000 yeast cells per apple, which is sufficiently low to track population dynamics of field and postharvest treated apples where values from 10^4 to 10^8 cfu/apple were obtained (Jijakli *et al*., 2002). Moreover, a threshold level of 10^4 cfu cm^{-2} of apple surface or 10^6 cfu/apple is required to obtain biological control (Jijakli *et al*., 2002) against mould diseases on cv. Golden Delicious apples. For higher population levels, sample dilutions should be considered to obtain a more accurate quantification. The estimation of very low natural population levels of strain K cells on apples remains difficult using this technique.

The sensitivity threshold of QC-PCR-ELOSA applied under practical conditions allowed detection of less than 10^3 yeast cells per apple. Using the same technique to recover yeast cells from treated apple surfaces, the sensitivity threshold of the plating method is around 10^5 cfu/apple (De Clercq *et al*., 2003). This molecular method achieves a much higher level of specificity in identifying strain K than does the plating method. It also gives quantification values of the same order of magnitude as those obtained by OD measurements of cells. The overestimation of the strain K cell numbers at low concentrations could result from low accuracy of the OD quantification method and manipulation errors when pipetting.

This method, based on quantitative PCR, has proved to be very specific and sufficiently accurate to monitor strain K populations. Manipulation time was considerably reduced by means of its automation procedure. However, in the near future, the development of a quantitative real-time PCR methodology will be considered in order to simplify post-PCR processing.

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