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Development of real-time PCR using Minor Groove Binding probe to monitor the biological control agent *Candida oleophila* (strain O)

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Abstract

A real-time PCR assay using a 3′-Minor Groove Binding (MGB) probe was developed for specific detection and monitoring of *Candida oleophila* (strain O), a biocontrol agent against *Botrytis cinerea* and *Penicillium expansum*, on harvested apples. The application of the RAPD technique on *C. oleophila* strains followed by reproducible sequence characterized amplified region (SCAR) amplifications allowed the identification of a semi-specific fragment of 244 bp, observed in the profiles of strain O and three other *C. oleophila* strains. After sequencing, polymorphisms (3%) were observed between the strain O sequence and the three other sequences. A 3′-Minor Groove Binding probe was designed to specifically match a region of the strain O sequence and was able to discriminate a single base mutation or a two-base difference in the corresponding sequences of the non-target strains. This specific detection method was applied to monitor strain O population, recovered by a washing buffer, from harvested apples. Population densities were calculated using an external standard curve consisting in a serial dilution of strain O cells in the washing buffer from untreated apples. Linearity in the standard curve was kept between 1.64×10^2 and 1.64×10^5 cfu cm^{-2} of apple surface. During a first practical experiment, the calculated population densities were similar to those obtained by plating on semi-selective media. This new real-time PCR method is a promising tool to monitor quickly and specifically strain O population on apple surface in middle- or large-scale experiments.

Keywords: Real-time PCR; Monitoring; *Candida oleophila*; Biological control

1. Introduction

Biological control of post-harvest pathogens is an emerging technology, which can overcome the main drawbacks of chemical control: fungicide resistant

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strains, deregistration of fungicides and public concerns about health and environmental impact (Arul, 1994; Franclet, 1994; Wellings, 1996). In this context, the yeast *Candida oleophila* Montrocher (strain O) has been isolated from apple (cv. Golden delicious) and selected for its high and reliable antagonistic properties against *Penicillium expansum* Link. and *Botrytis cinerea* Pers.:Fr, two of the most devastating pathogens of harvested apples (Jijakli et al., 1999). The transformation of a natural antagonism into an efficient and reliable biological protection strategy implicates numerous requirements. Among these, the monitoring of the released biocontrol agent has a crucial importance to understand and to predict the biocontrol efficiency in relation to modality of application, formulation and environmental conditions as the protective effect of strain O seems to be closely related to its colonisation on apples (Jijakli and Lepoivre, 1993). Moreover, it contributes to the establishment of a quality control procedure for the biofungicide and may facilitate the registration procedure.

A wide range of methods have been developed to monitor microbial populations. The dilution plate methods have been used successfully (Buck and Andrews, 1999; Johnson et al., 2000; Teixido et al., 1999) and have contributed to a better understanding of the population dynamic of microorganisms. Nevertheless, they present significant shortcomings which have led to the development of more specific and more rapid monitoring methods mainly based on DNA amplification (Olive and Bean, 1999; Soll, 2000). The methods based on conventional PCR allow the specific detection of the microorganisms but fail to quantify its population. Hence, they have already been combined with serial dilution plating (Abassi et al., 1999; De Clercq et al., 2003; Leibniger et al., 1997). The application of quantitative competitive PCR, like PCR-ELOSA, has enabled direct quantification of the microorganisms (Johnsen et al., 1999; Pujol et al., 2004). The competitive PCR has important drawbacks (time consuming, laborious protocol, cross-contamination of PCR products) and has been overtaken by the development of real-time PCR. Real-time PCR using the Taqman fluorescent chemistry has already been used to quantify microorganisms in their environment, like strains of *Candida* species in water samples (Brinkman et al., 2003), *Stachybotrys charatarum* in water and dust (Haugland et al., 2002) or plant pathogens in soil (Filion et al., 2003b). A more sophisticated Taqman probe, called 3′-Minor Groove Binder-DNA (MGB) probes, can be used to modify the detection specificity (Kutyavin et al., 2000; Marbot et al., 2003; Salmon, 2002).

The aim of this study was to develop a quantitative real-time PCR method for specific detection and monitoring of *C. oleophila* strain O. The paper describes the different steps involved in this development: (i) research of a DNA marker specific to strain O by the Random Amplified Polymorphic DNA (RAPD; Williams et al., 1990) followed by its transformation into a sequence characterised amplified region (SCAR; Abassi et al., 1999) marker; (ii) design and validation of a MGB Taqman probe for real-time PCR monitoring; and (iii) transfer of the optimised real-time PCR method to monitor strain O on apple.

2. Materials and methods

2.1. Microorganisms and media

Strains used in this study and their source are described in Table 1. For each experiment, strains were grown on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) at 25 °C. After three successive subcultures under the same conditions, strains were inoculated in YEPD (yeast extract 1% (w/v), peptone 2% (w/v), dextrose 2% (w/v); Merck) at 25 °C during 24 h before DNA extraction. For monitoring by plating, a semi-selective medium, composed of PDA supplemented with 12.5 mg l^{-1} hygromycine B (Duchefa, Haarlem, The Nederlands), 0.25 mg l^{-1} Thiram (UCB, Brussel, Belgium) and 5 mg l^{-1} Sumico (AgrEvo, Machelen, Belgium) was used (De Clercq et al., 2003).

2.2. DNA extraction

One ml of culture media or 1.5 ml of apple washing buffer (KPB buffer; 34 mM KH_2PO_4, 16 mM K_2HPO_4, 0.05% Tween 80) containing the yeast cells were centrifuged 15 min at 14 000 rpm. Yeast cells were suspended in 50 μl of 40 mM Tris–HCl pH 8 and 5 mM EDTA. One hundred microliters of lysis

Table 1
Yeast strains used and their source

Yeast species	Strain	Source
Candida oleophila	O	Plant Pathology Unit, FUSAGx (Gembloux, Belgium)
	CBS 2070	Centraalbureau voor Schimmelculturen (CBS, Utrecht, The Nederlands)
	CBS 2219	
	CBS 2220	
	CBS 4371	
	CBS 4704	
	CBS 7419	
	CBS 8269	
	I-182	Aspire™ (Ecogen, Langhorne, USA)
	ATCC 201074	American culture type collection (ATCC, Manassas, USA)
	ATCC 60367	
Candida sake	CBS 159	Centraalbureau voor Schimmelculturen (CBS, Utrecht, The Nederlands)
	CBS 2213	
	MUCL 4923	Mycothèque de l'Université Catholique de Louvain-La-Neuve (Belgium)
Pichia anomala	K	Plant Pathology Unit, FUSAGx (Gembloux, Belgium)
	J121	Department of Microbiology, Swedish University of Agricultural Sciences (Uppsala, Sweden)
	J375	
	J376	
	J381	
	J383	
Debaryomyces hansenii	MUCL 29030	Mycothèque
	MUCL 29907	de l'Université Catholique de Louvain-La-Neuve (Belgium)

solution (0.2 M NaOH, SDS 1%) were added and the sample was incubated at 100°C during 30 min with vortexing each 10 min. After cell lysis, 75 μl of 4 mM potassium acetate and 11.5% (v/v) of acetic acid were added before a 10-min incubation on ice. After another centrifugation step at 13,000 rpm during 5 min, the supernatant was ethanol precipitated. For monitoring strain O population on apple, the DNA purification step was improved by the use of the DNA purification protocol described in the DNeasy Kit (Qiagen, Venlo, The Netherlands) just after the step of cell lysis. Whenever possible, e.g. when detection limit was reached, the DNA was quantified by measuring the absorbance at 260 nm on an UltrospecII spectrophotometer (LKB Biochrom, Uppsala, Sweden).

2.3. RAPD analysis

One hundred random primers, corresponding to the kits K, L, M, N and O of the RAPD® 10mer kits series (Qiagen), were used (for complete sequence, see http://oligos.qiagen.com/stock/rapd_10mer_price.php). Fourteen primers (OPK 3, 4, 6, 9 and 19, OPL 11 and 14, OPM 9, 14 and 17, OPO 11, 16, 18 and 19) were tested on nine *C. oleophila* strains. A second screening was undertaken with the 86 other primers on strain O, CBS 2219 and CBS 2220. Each sample was amplified in a mixture of 50 μl containing 200 μM of primer (Operon technologies), 100 μM of each deoxynucleotide triphospate (dNTP), 1× PCR buffer containing 2.5 mM of $MgCl_2$, 1 Unit of Taq polymerase (Roche, Bazel, Switzerland) and 10 ng of genomic DNA. After a first cycle of 5 min at 95 °C, 4 min at 36 °C and 2 min at 72 °C, the PCR reaction was performed using 45 cycles at 94 °C for 15 s, 36 °C for 45 s and 72 °C for 90 s followed by a final extension step of 10 min at 72 °C. PCR products were subjected to electrophoresis in 1.5% agarose gel and revealed with ethidium bromide.

2.4. Computer analysis of RAPD results

For each strain, the presence or the absence of each band on RAPD profiles was determined and designated as 1 or 0, respectively. The generated distance matrix was used for hierarchical cluster analysis using the average linkage method with the Systat software version 5.0 (Systat, Evanston, USA). A dendrogram based on this analysis was constructed.

2.5. DNA purification, cloning and sequencing

RAPD fragments of interest were purified from agarose gel with Qiaex resin (Qiagen). The subsequent cloning step was performed using the TA cloning kit (Invitrogen, La Jolla, USA) according to manufacturer instructions. The Nucleobond AX kit (Macherey-Nagel, Düren, Germany) was used to isolate plasmids. Inserts were sequenced by Eurogentec (EGT Group, Liège, Belgium).

2.6. SCAR

Sequences obtained from selected RAPD fragments with the OPK19 primer were analysed by pairwise comparison using the PileUp software from the Wisconsin Package (Accelrys, Cambridge, United Kingdom). PileUp software creates a multiple sequence alignment using a method similar to the method described by Higgins and Sharp (1989). The six primers OF1, OF2, OF3, OR1, OR2 and OR3 were homologous to the internal sequence of one of the selected fragment, whereas OF4 and OR4 were homologous to its external sequence, obtained by inverted PCR (Ochman et al., 1988) (Table 2). The primer pairs were tested on the whole yeast collection listed in Table 1 under optimised PCR conditions [200 μM of forward and reverse primers (Invitrogen), 200 μM of each dNTP, 1×PCR buffer, 1.5 mM of $MgCl_2$, 1 Unit of Taq DNA polymerase (Roche), 10 ng of genomic DNA in a 50 μl final volume]. The thermal program consisted of an initial 5-min denaturation at 95 °C followed by 30 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s ended by 5 min at 72 °C.

2.7. Probe synthesis

Sequences obtained from the selected fragments amplified with the OF1/OR2 SCAR primers were compared by sequence alignment using the PileUp software. The MGB probe was designed using the software Primer Express 5.1 (PE Applied Biosystems, Foster City, USA) and following criteria of Livak, K., Marmaro, J. and Flood, S. (Perkin-Elmer Research News 57: 1–5) and Kutyavin et al. (2000). The MGB probe was supplied by Applied Biosystem with a 5′ covalently attached 6-carboxyfluorescine (FAM) reporter dye, a nonfluorescent quencher and MGB moiety at the 3′ end.

Table 2
Primers and probes designed for SCAR amplification and real-time PCR assay

Primers and probe	Sequence (5′–3′)	Size of primer (bp)
OPK 19	CACAGGCGGA	10
OF1	GGGAGTCAAACGTGAAAAAGAAGAAG	26
OF2	AGAATCGAAGCGCAGACGAC	20
OF3	CACAGGCGGAGAATCGAAGCG	21
OF4	TGAAGGTCGTCCAGGCGGAGAAT	23
OR1	GCCCCAATTAGGAAAATTATGCAAC	25
OR2	CTCTTCCACTTTGCTCATGGAAC	23
OR3	CACAGGCGGACCAGTAATGAAAG	23
OR4	AGCAGGAGGACCAGTAATGAAAGCT	25
MGB probe	TGAGTATTCGGAATGAGG	18

2.8. Real-time PCR

The GeneAmp 5700 (Applied Biosystem) was used for amplification and real-time quantification. Real-time PCR was performed in 25 μl volumes containing 1×PCR buffer (Eurogentec), 2.5 mM of $MgCl_2$, 0.5 unit of Hotgoldstar polymerase (Eurogentec), 200 μM of each dNTP, 400 μM of each primer, 200 μM of probe and 10 ng of DNA template. The optimised thermal cycle consisted of a denaturation step of 95 °C for 15 min followed by 40 cycles of 95 °C for 60 s and 60 °C for 60 s. To assess the specificity of the real-time PCR detection, genomic DNA, extracted from the whole collection of *C. oleophila* (Table 1) was subjected to real-time amplification in duplicate. Serial 10-fold dilution of strain O genomic DNA (500, 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 ng) were subjected in quadriplicate to real-time amplification to evaluate the sensitivity of the method.

2.9. Monitoring of strain O population density on apple surface

Golden delicious apples, harvested from an orchard untreated against post-harvest diseases, were soaked during 2 min in a water suspension containing 10^8 cfu of strain O ml^{-1}. Water was used as control. Apples were conserved 24 h at 20 °C and subsequently at 1 °C in two different rooms under normal atmospheric condition or under ultra low oxygen (ULO) conditions. Two repetitions of 25 fruits were used per treatment and storage condition. After 60 days, four apples per repetition were washed in 500 ml of KPB buffer during 20 min at 120 rpm. Both repetitions from the same storage room were pooled. The real-time PCR method was applied and compared to the dilution plate method (De Clercq et al., 2003). For plating, serial dilution series were prepared from 1 ml of KPB buffer and plated in triplicate. For real-time

PCR, two samples of 50 ml from each pool were separately filtered (0.45 μm, Supor®-450, Gelman Sciences, Ann Harbor, USA) and the cells were recovered in 1.5 ml of KPB buffer by vortexing during 2×10 min. Cells were subsequently subjected to DNA extraction. Real-time PCR was applied in quadriplicate for each sample, resulting in eight measures per treatment and storage condition. The external standard curve for determination of strain O population on apple surface consisted in duplicate 10-fold dilution series of O cells (from 1×10^2 to 1×10^8 cfu ml^{-1}) of which 1 ml was added to 49 ml of KPB buffer used to wash untreated apples. Cells were further subjected to filtration, DNA extraction and real-time analysis as above described.

3. Results

3.1. RAPD analysis

Among the bands observed for strain O during the first screening using 14 RAPD primers, none was strain O specific. The relatedness between the *C. oleophila* strains was evaluated by scoring a total of 159 bands and performing a cluster analysis (Fig. 1). Strains CBS 2219 and CBS 2220 differed from strain O for 14 and 16 of the 159 RAPD bands, respectively, whereas the higher difference was 74 bands observed between strain O and strain I 182. Strain O and its two closest strains, CBS 2219 and CBS 2220, were subjected to RAPD amplifications with the other 86 primers under the same PCR conditions. None of the 655 bands observed during this second screening was specific to strain O. In this context, a semi-specific fragment of 371 bp, reproducibly obtained with the OPK19 primer in the profiles of strain O and CBS 4704, was selected (Fig. 2). After cloning and sequencing, a pairwise comparison of strain O and CBS 4704 sequences using gap and end weighted gap analyses showed a sequence similarity of 40% and 36%, respectively. These results were used to design SCAR primers specifically homologous to strain O fragment.

3.2. SCAR amplification

The 12 primer sets (OF1/OR1, OF1/OR2, OF1/OR3, OF1/OR4, OF2/OR1, OF2/OR2, OF2/OR3, OF3/OR1, OF3/OR2, OF3/OR3, OF4/OR2 and OF4/OR4) failed to identify a specific SCAR fragment for stain O. However, a semi-specific fragment of 244 bp was reproducibly observed with the OF1/OR2 primer pair for strains O, CBS 2219, CBS 2220 and CBS 4371 at an annealing temperature of 60 °C (Fig. 3), even though repetitive RAPD profiles did not reveal the 371-bp fragment for these three strains. Otherwise, the strain CBS 4704, which shared the RAPD frag-

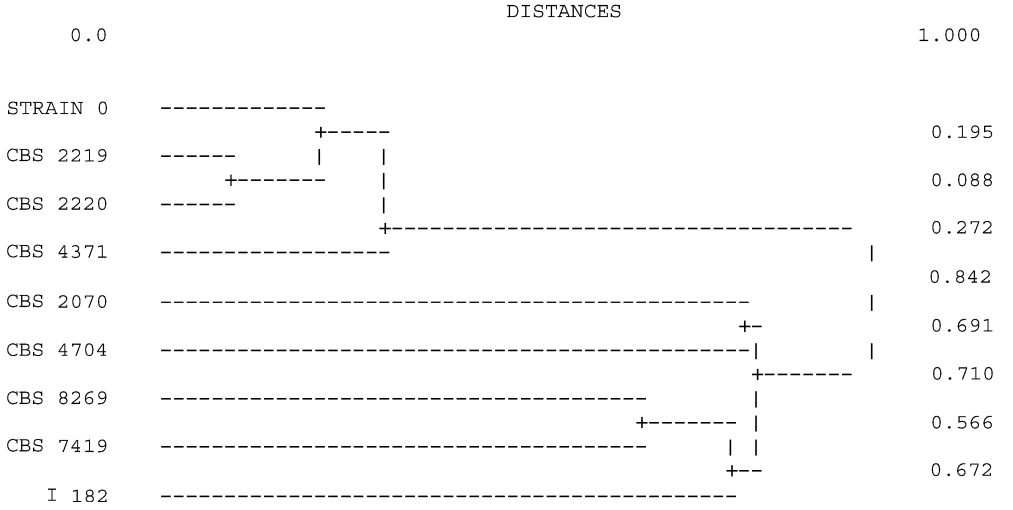

Fig. 1. Dendrogram based on the presence or absence of 159 bands in the profile of 9 *C. oleophila* strains. Distances correspond to the proportion of unshared RAPD bands.

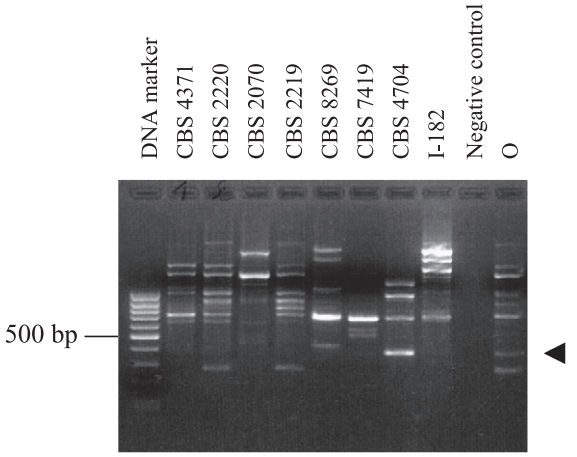

Fig. 2. RAPD fingerprinting of *C. oleophila* strains with OPK19 primer. The black arrow indicates the size of the semi-specific fragment. DNA marker: Generuler™ 100 bp DNA ladder (Fermentas). Negative control: addition of sterile water to the PCR mix.

ment with strain O, the other *C. oleophila* strains and the strains of *P. anomala*, *C. sake* and *D. hansenii* listed in Table 1 did not present amplified fragment with the OF1/OR2 primers pair tested (unpresented results). Different annealing temperatures (65, 66 or 67 °C) and times (20, 30 or 45 s) were tested to improve the specificity of this primer pair but none led to a better specificity (unpresented results).

3.3. Sequence alignment and probe design

After cloning and sequencing the PCR product of each strain, the forward and reverse sequences of each DNA fragment were aligned. An identity of at least 97% was calculated between the sequence of strain O and the respective sequences of strains CBS 2219, CBS 2220 and CBS 4371. A region located between position 130 and 150 presented a particular interest (Fig. 4). Two single nucleotide polymorphisms (SNP) were observed between strain O and the strains CBS 2200 and CBS 4371 at positions 134 and 138. Furthermore, another SNP was detected between strain O and strain CBS 2219 at position 144. A MGB probe, potentially specific to strain O sequence, was designed. The SNP of strain CBS 2219 was located in the MGB recognition region and the two SNPs of strains CBS 2220 and CBS 4371 were located near the 5′ end of the probe.

3.4. Real-time detection of strain O

A specific signal for strain O was reproducibly obtained with the use of the designed MGB probe together with the Hotgoldstar polymerase and an annealing temperature of 60 °C (Fig. 5). In opposite, a unique band was detected for strain O and the strains

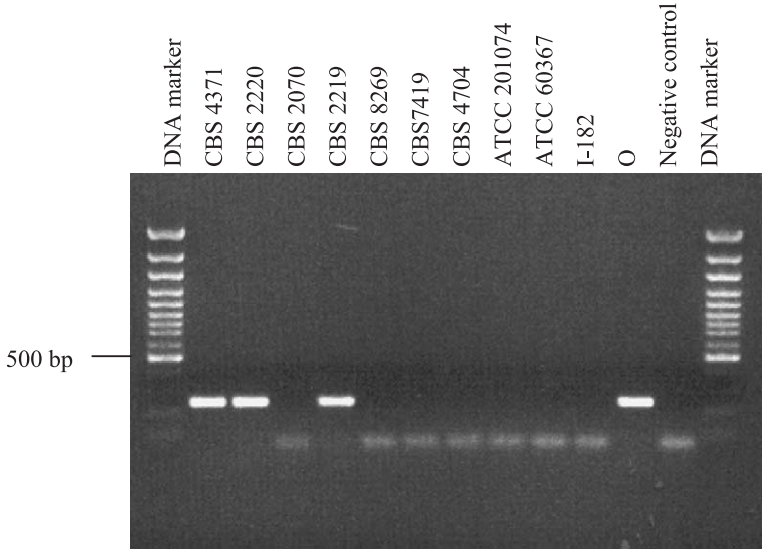

Fig. 3. Amplification of a SCAR marker semi-specific to strain O by the use of OF1/OR2 primer pair. DNA marker: Generuler™ 100 bp DNA ladder plus (Fermentas). Negative control: addition of sterile water to the PCR mix.

O	126	TTTTTGAGTATTCGGAATGAGGGAGGTTAT	157
CBS 2219	126	.....C.....	157
CBS 2220	126	.....C...A.....	157
CBS 4731	126	.....C...A.....	157

Fig. 4. Design of a 3′ MGB DNA probe specific to strain O sequence. The broader black line correspond to the DNA sequence of the probe, R: reporter, Q: quencher, MGB: Minor Groove Binder (represented by the thinner black line).

CBS 2219, CBS 2220 and CBS 4371 when PCR products were revealed on agarose gel (unpresented results). No band was observed for the other *C. oleophila* strains and the negative control on that gel. These results were identical to those obtained during SCAR amplification using the same conditions. The standard curve calculated on the serial dilution of genomic DNA (from 500 to 0.01 ng) was: [Ct value]=−3.29×[log(genomic DNA concentration)]+29.97 (r=0.99). A linear relationship was observed between Ct values and DNA concentrations for every tested concentration. The lowest DNA concentration tested (0.01 ng) showed a Ct value of 35.8.

3.5. Practical application of real-time PCR

The real-time PCR method was applied to determine the population density of strain O in two atmospheric conditions after a two-month storage period. The obtained results for the standard curve were [Ct value]=−3.17×[log(strain O cells)]+51.36 (r=0.96). The detection limit was 10^4 cfu (50 ml)$^{-1}$ of KPB buffer with an average Ct value of 38.16, which corresponds to 1.64×10^2 cells cm^{-2} of apple skin. No signal amplification was detected for the untreated controls. The calculated values were similar although slightly inferior to the population densities obtained by plating (Table 3).

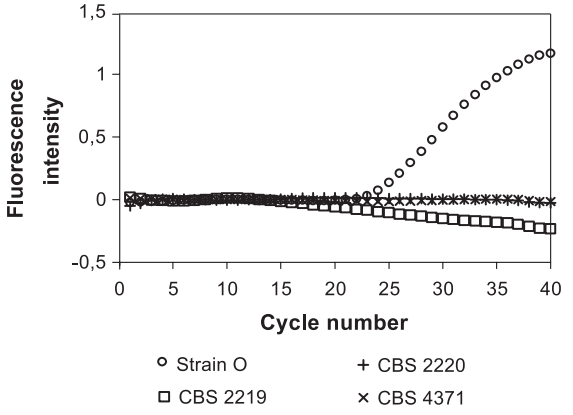

Fig. 5. Fluorescence intensity obtained during the amplification of the semi-specific SCAR marker using a MGB probe matching specifically the strain O sequence and comparison with signals obtained with strains CBS 2219, 2220 and 4371.

4. Discussion

We report the development of a real-time PCR method using a MGB probe which allowed specific detection of strain O while a semi-specific PCR fragment was detected for strain O and three other strains of *C. oleophila* on agarose gel after PCR using the same primer pair. This real-time method was used to monitor strain O population in its applied environment, the harvested apples.

The RAPD, already successfully used to discriminate a specific yeast strain from other natural strains present in the environment (De Clercq et al., 2003; Psomas et al., 2001), failed to identify a specific DNA fragment for strain O. A cluster analysis based on RAPD results showed a close relatedness between

Table 3
Comparison of population density of strain O on apples obtained by serial plate dilution (based on six values) and by real-time PCR (based on eight values) in two storage conditions: atmospheric (Atm) and ULO

Sample	Method of quantification		
	Real-time PCR		Plate dilution
	CT values± S.D.	cfu cm^{-2} of apple skin±S.D.	cfu cm^{-2} of apple skin±S.D.
Atm	31.59±0.75	$2.1\times10^4\pm0.9\times10^4$	$2.5\times10^4\pm0.3\times10^4$
ULO	32.26±0.81	$3.3\times10^4\pm1.6\times10^4$	$4.4\times10^4\pm0.8\times10^4$

Means and standard deviation (S.D.) are exposed for each sample.

strain O and strains CBS 2219, CBS 2220 and, to a lower extend (26 different bands on 159), CBS 4371. As also observed by Quesada and Cenis (1995), the ability of RAPD to discriminate closely related strains may be limited. Other PCR methods, like amplification of the internal transcribed spacers I and II, the ERIC-PCR and the REP-PCR, were applied to discriminate strain O from the other strains but also failed to identify a specific marker for strain O (unpresented results). A semi-specific RAPD fragment was selected and sequenced to design SCAR primers. These SCAR primers were tested on 11 strains of *C. oleophila*, corresponding to the majority of strains available worldwide in the public collections, and on 11 strains of other yeasts commonly isolated from apple surface (Table 1). The use of OF1/OR2 primers allowed the amplification of a semi-specific SCAR fragment of 244 bp for strains O, CBS 2219, CBS 2220 and CBS 4371. This result was consistent with the high similarity observed between these four strains after hierarchical cluster analysis (Fig. 1).

To identify a specific DNA fragment for real-time analysis, other DNA fingerprinting methods could be used but several reports (Afonina et al., 2002; de Kok et al., 2002; Jurevic et al., 2003; Shi, 2001) underlined the capacity of a real-time PCR assay using DNA probes to reveal a unique base mutation in the targeted DNA sequence. Compared to molecular beacons and Scorpion probes, Taqman and MGB probes provided a simple and straightforward design. The presence of a MGB stabilises the duplex between the probe and its target by folding into the minor groove formed by the terminal 5–6 bp of the duplex. The addition of a MGB conjugate can increase the specificity of a probe, especially when a mismatch is situated in the MGB region, but also its melting temperature (Kutyavin et al., 2000). This second property allows the design of shorter probes, which maintain an optimal melting temperature. Due to their small size, these probes are more specific for single base mismatches and fluorescence quenching is more efficient, resulting in an increased sensitivity (Kutyavin et al., 2000, Salmon, 2002). For these reasons, the covalent conjugation of the MGB moiety to the Taqman probe was preferred over the classical Taqman probe. A MGB probe was designed to match the semi-specific SCAR fragment of strain O from positions 131 to 148. This probe was able to discriminate a single base mutation in the corresponding sequence of strain CBS 2219 as well as the two-base difference observed in the corresponding sequences of strains CBS 2220 and CBS 4731 (Fig. 3). During the development of a strain O monitoring method, the specificity was brought by the detection method rather than by the use of specific primers.

Beside unambiguous detection, monitoring strain O population on apple surfaces requires quantification on apple surface. For this purpose, an external standard curve was drawn by serial dilution of strain O cells in KPB buffer from washed untreated apples. In comparison with serial dilution of genomic DNA (Filion et al., 2003a), plasmid standard (Bach et al., 2003) or cells (Bowers et al., 2000) in sterile water, this protocol fits better to the whole sample processing and takes into account the unavoidable minimal inaccuracies occurring during DNA extraction and manipulation (e.g. presence of PCR inhibitors). A new standard curve is drawn for each new DNA extraction and amplification. The calculated slopes are above the theoretical maximal value of −3.32 although a unique band was observed on agarose gel for the strain O samples and no band was observed for the negative control. The origin of this phenomenon, already observed in the literature (Casini Raggi et al., 1999; Leutenegger et al., 1999; Wang et al., 2004) is still unknown. Linearity in the standard curve was kept between 1.64×10^{2} and 1.64×10^{5} cfu cm^{-2} of apple surface. Beyond these values, linearity was lost or no signal was detected. However, the maximal concentration of strain O cells observed during large-scale experiments ($5\cdot10^{4}$ cfu cm^{-2}) (De Clercq et al., 2001) is included in the linear range of the method. Furthermore, the detection limit is 60-fold lower than the lowest initial concentration (10^{4} cfu cm^{-2} of apple surface) required for an efficient biocontrol of the pathogens (De Clercq et al., 2001). These values make the method suitable to check the establishment of strain O population on apple just after its application and to monitor its population during the whole conservation period.

The population density values obtained by both methods in ULO condition indicated that strain O seems well adapted to these modern storage conditions. On the other hand, fair differences were observed between the average population densities obtained by real-time PCR and those obtained by plating on semi-selective media which has been already used to monitor strain O population on apple

surface (De Clercq et al., 2001). Such similarities were also obtained by Brinkman et al. (2003) with some *Candida* species. Nevertheless, the population densities obtained by both methods are difficult to compare. A thorough analytical comparison, including more repetitions would be required for accurate comparison between both methods.

In our experiments, this real-time PCR method has been the only method able to specifically identify strain O from the other strains thanks to the use of a MGB probe. It represents a novel potential tool to monitor more quickly and specifically strain O population on apple surface in large-scale experiments but also in commercial conditions, in comparison with other classical techniques.

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Massart, S. and De Clercq, D. have equally contributed to this paper.

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