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Development of a RAPD marker and a semi-selective medium for *Aureobasidium pullulans* (strain Ach1-1), a biocontrol agent against post-harvest diseases on apples

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Abstract: *Aureobasidium pullulans* strain Ach1-1 is an efficient biocontrol agent against *Botrytis cinerea* and *Penicillium expansum* on apples. Specific tools have to be developed in order to monitor this biocontrol agent. This will contribute to evaluate its adaptation and its survival ability on post-harvest commodities or to assess the effectiveness of various application methods. The monitoring of the antagonist has been achieved following two complementary approaches. On the one hand, a specific RAPD marker (522 pb) to strain Ach1-1 has been obtained among a collection of 11 strains of *A. pullulans* with primer OPR-13. This fragment was cloned and sequenced in order to develop a SCAR marker for the strain Ach1-1. On the other hand, a semi-selective medium for quantification of strain Ach1-1 is under development. This medium presents a high toxicity towards the air microflora, while the growth of strain Ach1-1 was unaffected.

Keywords: monitoring, SCAR

Introduction

The yeast-like fungus *Aureobasidium pullulans* (De Bary) Arnaud strain Ach1-1, Ach2-1 and Ach2-2 were isolated in the Plant Pathology Unit of F.U.S.A.Gx (Belgium) from *Golden Delicious* for their high antagonistic activity (more than 80%) against *Botrytis cinerea* and *Penicillium expansum,* two major pathogens of post-harvest apples (Achbani et al., 2005).

A. pullulans strains are currently investigated for their biological control ability on various fruit and vegetable models (Ippolito et al., 2000; Schena et al., 1999). However, among the multiple stages leading to the commercialization of a biopesticide, the monitoring of the strain constitutes an important step. Monitoring tools are required to specifically identify the studied strain among a complex population of microorganisms. Furthermore, it will allow tracking the population evolution according various application techniques or formulations under different environmental conditions. Several methods were developed to follow population dynamic of biocontrol agents. A technique combining dilution plating on a semi-selective medium and strain-specific identification with a SCAR (Sequence Characterized Amplified Region) marker was developed by De Clercq et al. (2003). This technique was recently improved by the use of real time PCR, allowing the combination of SCAR and Taqman fluorescent chemistry (Massart et al., 2004).

In this study, RAPD (Random Amplified Polymorphic DNA) was used for identification of a specific molecular marker for *A. pullulans* starin Ach1-1. This specific fragment was

sequenced in order to obtain a SCAR marker specific to the strain Ach1-1. A semi-selective medium for the strain Ach1-1 was also developed.

Material and methods

Aureobasidium strains and culture conditions

A. pullulans strains used in this study are described in Table 1. Before each experiment, the strains were grown on potato Dextrose Agar (PDA) (Merck, Germany) at 25°C during 48 h, for three successive generations. For DNA extraction, strains were inoculated into YEPD (yeast extract 1%, peptone 2%, dextrose 2%; Merck) at 25°C over 24 h with agitation.

Table 1. Origin and source of *A. pullulans* strains

Yeast	Strains	Original Substrate	Source
A. pullulans	Ach1-1	apple, Golden Delicious (Belgium)	Institut National de Recherches Agronomiques-Meknès (Morocco)
	Ach2-1		
	Ach2-2		
	MUCL 22377	Leaves; *Quercus robur* (Sweden)	Mycothèque de l'Université Catholique de Louvain-La-Neuve (Belgium)
	MUCL 20326	Leaves; *Hordeumsativum* (Belgium)	
	MUCL 20322	tiles (The Netherlands)	
	MUCL 6147	*Picea abies* (Norway)	
A. pullulans var. pullulans	MUCL 7862	Cupule; *Fagus sylvatica* (Belgium)	
A. pullulans var. melanogenum	MUCL 8724	soil (Zaire)	
	MUCL 19714	fruit; *Symphoricarpos rivularis* (Sweden)	
	MUCL 19360	tree: *Xyloterus lineatus* (Belgium)	
	MUCL 43163	Leaves and stem; *Citurs sinensis* (Brazil)	

DNA extraction and RAPD analysis

A. pullulans genomic DNA was isolated according to Ausubel et al. (1987).

The RAPD primers used in this study were obtained from Operon Technologies Inc. (Alameda, California). Thirty nine random primers were tested on extracted DNA of 12 *A. pullulans* strains including strain Ach1-1. The PCR reactions were performed in 25 µl mixture containing 1.0 U of Taq DNA polymerase (Sigma-Aldrich, Inc., USA), 2.5 µl of 10× PCR buffer Sigma, 100 µM of each dNTP, 2.5 µl of $MgCl_2$, 0.5 µM selected RAPD primer and 50 ng of genomic DNA. PCR amplifications started with an initial denaturation at 94°C for 5 min, followed by 44 cycles at 94°C for 15 s, 36°C for 45 s and 72°C for 90 s with a final extension step at 72°C for 10 min. Aliquots (10 µl) of PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by staining with 1 µg/ml ethidium bromide.

Cloning of strain-specific RAPD marker

The specific RAPD fragment was isolated from the gel with QIAEX II DNA Purification Kit (Qiagen) according to the manufacturer's instructions This Fragment was cloned using the TA Cloning kit (Invitrogen, USA). The Ultraprep plasmid DNA (AHN Biotechnologie, Germany) was used to isolate plasmids. The inserts were sequenced in both directions using the BigDye

Terminator 3.1 cycle sequencing Kit (Applied Biosystems). The sequence obtained was analyzed for potential similarity with sequences in GenBank database using BLASTn.

Semi-selective medium

Development of a semi-selective medium for the strain Ach1-1 was carried out by various combinations of five fungicides (Sumico, Lirotect, Thiram, Topsin, Euparen), and six antibiotics (Hygromycin B, streptomycin sulfate, spectinomycin, tetracyclin, cyclohiximide, Geneticine). Strain Ach1-1 (10^2 ufc) were plated out on each medium (four replicates for each medium) and incubated at 25°C for 7 days. The selectivity of the medium was evaluated against the natural laboratory microflora. Petri dishes were opened during three hours in the laboratory. After one week of incubation at 25°C, colonies were visually observed and enumerated.

Results

Identification of strain-specific RAPD markers

Thirty nine random primers were first tested with strain Ach1-1 DNA and two other strains Ach2-1 and Ach2-2. Eight primers were selected (OPP-01, OPQ-04, OPQ-12, OPR-06, OPR-13, OPT-01, OPT-05, OP-T17) for their ability to generate at least one specific DNA fragment to the strain Ach1-1. The size of specific bands was ranging between 2.6 and 0.4 kb. Selected primers were then evaluated on the complete *A. pullulans* collection, fragments specific to strain Ach1-1 were obtained with five primers (Table 2).

Table 2. Size and number of the specific bands to the strain Ach1-1

Primers	Sequences	Number of specific bands	Size of specific bands (bp)
OPQ-04	5'-AGTGCGCTGA-3'	1	1060
OPQ-12	5'-AGTAGGGCAC-3'	1	875
OPR-13	5'-GGACGACAAG-3'	1	525
OPT-01	5'-GGGCCACTCA-3'	1	525
OPT-17	5'-CCAACGTCGT-3'	1	1300

The RAPD patterns obtained with the primer OPR-13 are presented in Fig. 1. Eight to twenty two DNA fragments ranging from 0.4 to 3.2 kb were amplified. The Ach1-1 specific fragment was cloned and sequenced, leading to a 522 bp sequence. Nucleotide homology searches revealed no appreciable similarity with sequences in the GenBank database.

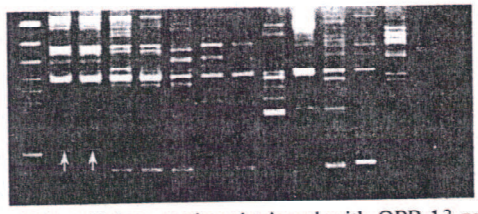

Figure 1. RPAD patterns of *A. pullulans* strains obtainned with OPR-13 primer. Ach 1-1 (1 and 2); Ach 2-1 (3) ; Ach 2-2 (4); MUCL 22377 (5); MUCL 20326 (6); MUCL 20322 (7); MUCL 6147 (8); MUCL 8724 (9); MUCL 7862 (10); MUCL 19714 (11); MUCL 19360 (12); MUCL 43163 (13); Negative control (14); M: DNA marker: Generuler™ 100 bp DNA ladder plus (Fermentas); The white arrow indicates the specific fragment.

Semi-selective medium

Fungicides and antibiotics were combined and the toxicity was evaluated as described in material and methods. The results of some non-toxic combination (% of viability superior to 90%) for the strain Ach 1-1 are represented in Table 3.

Table 3. Analysis of medium toxicity on the growth of *A. pullulans* strain Ach1-1 in comparison to the PDA medium

Code	Concentration of fungicides and antibiotics	% of viability ± standard deviation
S2	L 10ppm + T 1ppm + SP 25ppm + TR 50ppm	107.8 ±16.76
S16	S 1.5ppm + T 1.5ppm+ ST 30ppm +G 2.5ppm	101.37±19.24
S17	S 1.5ppm + T 1.5ppm+ TR 50ppm +G 2.5ppm	102.73±16.36
S18	E 0.5ppm + S 1ppm+ H 2.5ppm + ST 30ppm	102.74±13.21

Lirotect (L), Thiram (T), Sumico (S), Euparen (E), Hygromycin B (H), streptomycin sulfate (ST), spectinomycin (SP), Tetracyclin (TR), cyclohixymide (C), Geneticine (G)

Selectivity of these combinations was then evaluated on the air microflora. Only the S18 medium showed an important inhibition for the air microflora, allowing the sole development of one small and white filamentous fungus, while PDA medium was at the same time covered with several types of filamentous fungi (Fig. 2).

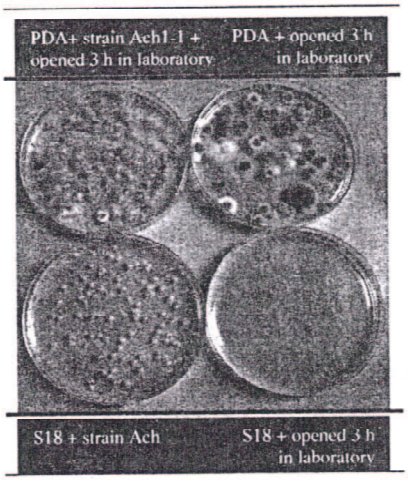

Figure 2. Toxicity test of the S18 semi-selective medium against strain Ach1-1 and air microflora

Discussion

We described here the development of a monitoring technique specifically adapted to strain Ach1-1 of *A. pullulans*, previously isolated for its biological control properties against *P. expansum* and *B. cinerea* combining molecular and microbiological detection tools, our approach will allow an easy quantification of the biological control agent after application but also its accurate identification among various strains of the same species.

Considered as a rapid and simple technique to study genetic polymorphism with population of the same species (Olive & Bean, 1999), RAPD was used here in order to identify a SCAR marker specific to strain Ach1-1.

The specific fragment obtained with the RAPD primer OPR-13 was cloned and sequenced, generating a 522 bp fragment. Starting from this specific sequence, our objective is now to identify a SCAR sequence compatible with real time PCR requirements. Beside the direct quantification of the strain, Real time PCR really improved the rapidity, the sensitivity and the accuracy of the PCR-based detection techniques (Massart et al., 2004).

Our monitoring system also relies on a semi-selective medium, allowing simple identification of the biological control agent by a classical dilution plating method. Despite its lower accuracy, this technique remains an efficient alternative to molecular methods. The medium developed here prevents the growth of air microflora and was not toxic for strain Ach1-1. In further studies, the specificity of our medium will be evaluated against the washing water of apples. This point is of major importance since the semi-selective medium will be used to quantify the antagonist population on apple surface.

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