Postharvest Biology and Technology 51 (2009) 227–231

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Real time polymerase chain reaction for rapid and quantitative determination of *Cystofilobasidium infirmominiatum* on the surfaces of apple, pear, and sweet cherry fruit

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ARTICLE INFO

Article history:
Received 30 April 2008
Accepted 23 July 2008

Keywords:
Biological control
Detection
Malus sylvestris
Prunus avium
Pyrus communis
Real time PCR

ABSTRACT

The objectives of this study were to develop primers and a real time PCR protocol for the postharvest biocontrol yeast *Cystofilobasidium infirmominiatum* (*Cim*). The application of this technology was developed to quantify *Cim* on the surfaces of apple, two pear cultivars, and sweet cherry fruit treated over a range of concentrations. Statistically significant relationships were observed between *Cim* DNA on fruit surfaces, expressed as μg/m^2, and CFU/L of dip suspensions for apple, pear, and sweet cherry. In addition, the relationship for each fruit was significantly different from the other three fruits. Threshold values of concentrations of *Cim* DNA on the fruit surface were calculated based on regression equations and a dose of 2.0×10^{11} CFU/L of dip suspension, the dose for optimum decay control, and were 4.8, 7.0, 16.5, and 25.2 μg/m^2 for Bosc pear, Lapins sweet cherry, d'Anjou pear, and Golden Delicious apple, respectively. Monitoring *Cim* DNA concentration on fruit surfaces will assure that *Cim* is being properly applied to fruit and that a sufficient number of cells are present for optimum decay control.

1. Introduction

In 1993, the naturally occurring saprophytic yeast *Cystofilobasidium infirmominiatum* (Fell, I.L. Hunter & Tallman) Hamam., Sugiy. & Komag YY6 (anamorph: *Crytococcus infirmominiatus* (Okunuki) Phaff et Fell) (*Cim*) was isolated from the surface of a Bartlett pear in Yakima, WA, USA (Chand-Goyal and Spotts, 1996a). *Cim* is an effective biological control agent for a variety of postharvest crop/pathogen systems including gray mold, side rot, mucor rot, blue mold, bull's-eye rot of pear (Chand-Goyal and Spotts, 1996b, 1997), blue mold of apple (Chand-Goyal and Spotts, 1996c, 1997), and brown rot and blue mold of sweet cherry (Chand-Goyal and Spotts, 1996c; Spotts et al., 1998; Spotts et al., 2002).

The concentrations of yeasts on plant surfaces have been studied extensively, and methods of removal include swabbing (Marshall and Walkley, 1951), washing (Kamra and Madan, 1987), blending (Buhagiar and Barnett, 1971), and ultrasound (Guerzoni and Marchetti, 1987). We determined that the combination of rotary shaking and sonication was optimum for removal of yeasts from fruit surfaces, and then used this protocol to isolate *Cim* and other yeasts from pear fruit in Oregon and Washington (Chand-Goyal and Spotts, 1996a).

Effectiveness of biological control yeasts is closely related to the concentration of yeast cells in the treatment suspension (Chand-Goyal and Spotts, 1996b; Fan and Tian, 2001; Filonow et al., 1996; He et al., 2003). We found that it is necessary to apply 2×10^{11} cells of *Cim* per liter for effective control of decay (Spotts, unpublished data). Yeast enumeration and population dynamics traditionally involves dilution plating, then counting colonies after 2 or 3 d of incubation (Fan and Tian, 2001; Leibinger et al., 1997; Lima et al., 1998; Usall et al., 2000).

Recently, quantitative real time polymerase chain reaction (qPCR) has been used to quantify a wide range of fungi (Gachon et al., 2004; Schena et al., 2004). We sequenced a 581 base pair sequence of the ITS region of the ribosomal RNA genes as a preliminary step to developing PCR primers for quantification of *Cim* (Spotts et al., 2003). The objectives of this study were to (1) develop *Cim* primers and a qPCR protocol and (2) use these molecular tools to quantify *Cim* on the surface of apple, pear, and sweet cherry fruit treated with a range of concentrations. Threshold values are the concentration of *Cim* DNA or the number of *Cim* cells that will be present per square meter of surface area when the optimum dose of *Cim* in suspension is properly applied to the fruit. Threshold values of *Cim* DNA and number of *Cim* cells per square meter of fruit

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doi:10.1016/j.postharvbio.2008.07.015

surface were established to assure that *Cim* is applied properly to fruit and that a sufficient number of cells are present for optimum decay control.

2. Materials and methods

2.1. Preparation of Cim suspension

Cim was removed from storage at −70 °C and transferred to yeast malt dextrose agar (YMDA) petri dishes as described previously (Spotts et al., 1998). After 48 h of incubation at 23 °C, *Cim* cells were removed with a sterile spatula, suspended in sterile distilled water (SDW), centrifuged twice at $1.06 \times 10^4 \times g$ for 15 min, each time discarding the supernatant. After the second centrifugation, the cells were re-suspended in SDW, and the concentration was adjusted to 1% transmittance at 550 nm using a spectrophotometer (Spectronic 20, Bausch and Lomb Optical Co., Rochester, NY). This resulted in a yeast concentration of $2.5 \times 10^{11} \pm 1.8 \times 10^{11}$ CFU/L (CFU: colony-forming units). Yeast concentration was verified by dilution plating on YMDA. The concentrated yeast suspension was diluted twice with an equal volume of SDW each time to obtain suspensions of full, half, and quarter strength for inoculation of fruit. SDW without *Cim* cells was included as the control.

2.2. Application of Cim to fruit

Healthy Golden Delicious apple, Bosc pear, d'Anjou pear, and Lapins sweet cherry fruit were harvested at commercial maturity in 2006 and 2007 from trees that had not been sprayed with fungicides during the growing season. All fruit were surface-sterilized with sodium hypochlorite, 100 mg/L, and then rinsed with tap water. In addition, sweet cherry fruit were dipped in 95% ethanol and triple rinsed with tap water. Apple and pear fruit were held by the stem and individually dipped into *Cim* suspensions. Sweet cherry fruit were placed in a wire basket and dipped in groups of 12. For each of the three *Cim* concentrations and the SDW control, three replicates consisting of five apple or pear and 12 sweet cherries per replicate were dipped in *Cim* suspensions. Inoculated fruit were placed on screens in a laminar flow air bench (EdgeGuard hood, The Baker Co., Inc., Sanford, Me) to dry for 1–2 h before cell removal.

2.3. Fruit washing and DNA extraction

Inoculated fruit were weighed, washed on a rotary shaker followed by sonication (Lennox et al., 2003), and the surface area of each d'Anjou pear and apple fruit was estimated from a regression equation (Chen et al., 1990) with fresh weight as the independent variable and for each Bosc pear fruit from the equation $Y = 0.651X + 56.0$, where Y = surface area in cm^2 and X = fruit weight in grams. Surface area (Y) of sweet cherry fruit was calculated as the surface of a sphere where $Y = \pi d^2$ with d as fruit diameter. *Cim* cells in wash water were concentrated by successive centrifugation starting with 200 mL of original wash water that was concentrated to 10 mL and finally to 100 μL at 167 s^{-1} for 15 min, 117 s^{-1} for 15 min, and 100 s^{-1} for 1 min, respectively. Total *Cim* cell concentration in the 10 mL volume was determined by removing 500 μL and dilution plating on YMDA. *Cim* CFU/m^2 were calculated from total cell counts and the fruit surface area. Genomic DNA was extracted from 10 μL of the final concentrate using the UltraClean™ Soil DNA Kit (MoBio, Solana Beach, CA) following the manufacturer's instructions. DNA was stored at −70 °C and analyzed with qPCR within 1 month.

2.4. DNA sequencing and primer design

The primers ITS 5 and ITS 4 (White et al., 1990) were used in separate reactions with the Big Dye Terminator sequencing mix (Applied BioSystems, Foster City, California) to sequence the ITS 1, 5.8S and ITS 2 rDNA regions of *Cim*. The sequencing reactions were performed in a GeneAmp 2400 programmed for 25 cycles of 96 °C for 10 s, followed by 50 °C for 5 s, and an extension at 60 °C for 2 min. Extension products were precipitated in the presence of 1/10 volume of 3 mol/L sodium acetate, 3 volumes of 95% ethanol, incubated at room temperature for 15 min, and centrifuged at $12{,}000 \times g$ for 20 min. The pellets were washed in 70% ethanol and air dried on the bench top. Electrophoresis of extension products was performed on an ABI 310 sequencer (Applied BioSystems, Foster City, CA). Sequence data were edited using Sequence Navigator software (Applied BioSystems, Foster City, CA). Ambiguities identified in the sequence data were resolved by comparison with reverse complimentary sequences. Multiple sequences for *C. infirmominiatum* strain YY6, were used to generate a consensus sequence which was deposited in Genbank (Spotts et al., 2003).

Alignments with *C. infirmominiatum* rDNA and other closely related sequences obtained from Genbank were performed using Clustal X software (SGI, Mountain View, CA). PCR primers sequences for *C. infirmominiatum* rDNA were selected from the alignments. Selected forward and reverse primers were CIM87 5′-CGC TTC ATT GCG GTG GTC-3′ and CIM450 5′-CGT CGA GTA GAA CCA ACA C-3′, respectively.

2.5. Real time PCR protocol

Twenty-microliter samples were prepared by mixing 1 μL DNA solution with 6.8 μL SDW, 10 μL DyNAmo HS SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland), 1 μL of each primer mentioned previously (final concentration 200 nmol L^{-1}) and 0.2 μL UltraTherm DNA polymerase (Boca Scientific, Boca Raton, FL). Real time PCR reactions were run in triplicate on a DNA Engine Opticon 2 System (MJ Research Inc., Waltham, MA). After a 10-min denaturation step at 95 °C, samples were run for 40 cycles of 10 s at 94 °C, 20 s at 53 °C and 15 s at 72 °C. A final extension was done at 72 °C for 10 min. Melting curves were measured starting at 75 °C and increasing by 0.2 °C every second until 90 °C was reached. Positive controls (standards) consisted of DNA extracted from *Cim* and quantified using a spectrophotometer, reading transmittance at 260 nm and 280 nm. Five serial 10-fold dilutions of these standards were run in triplicate in the same reaction as the samples. Three negative controls, consisting of 1 μL SDW added to the qPCR mix in place of the DNA were included per run.

2.6. Statistics

A regression analysis procedure (Minitab, State College, PA) was used to determine significance among regressions and whether or not differences in slope and Y intercept were present that would prohibit combination of data from the 2006 and 2007 repeated experiments. The procedure was performed with DNA/m^2 or cells/m^2 of fruit surface as the response variable Y, group as year, and the covariate X as inoculum dose in the dip suspension. A significant P value of the coefficient for the interaction term of group and X indicated that the slope coefficients for the 2 years were significantly different. When the slope difference was not significant, the interaction term was deleted and the regression rerun. A significant P value (less than 0.05) of the coefficient for the group variable indicated that the intercepts of response variables for the 2 years were significantly different.