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Molecular tools applied to identify and quantify the biocontrol agent *Pantoea agglomerans* CPA-2 in postharvest treatments on oranges

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ABSTRACT

Pantoea agglomerans strain CPA-2 is an effective biocontrol agent (BCA) for postharvest diseases of citrus and pome fruit. To implement their use as a control strategy is necessary to study the traceability of BCAs in the environment during application, for registration issues. In this study, the presence and persistence of CPA-2 was monitored in the packing line, storage chambers and on working clothes by conventional PCR. After postharvest application, the presence of CPA-2 was not detectable in the environment and storage chambers, whereas on working clothes and the packing line its persistence was less than 1 and 3 days, respectively. Additionally, the CPA-2 population was quantified on oranges stored at two different temperatures (20 °C and 4 °C) by quantitative PCR (qPCR), sample pretreatment with a propidium monizade dye (PMA–qPCR) and the dilution plating method. At the initial time of the assay, no differences were observed in CPA-2 populations quantified by qPCR, PMA–qPCR, and dilution plating, at both storage temperatures. However, CPA-2 populations quantified by PMA–qPCR were significantly different compared with those obtained by qPCR during the time-course of the assay; no significant differences were observed between PMA–qPCR and dilution plating. In conclusion, the persistence of *P. agglomerans* CPA-2 at different sampling areas after postharvest application was low. Furthermore, PMA–qPCR gave valuable information on viable population behavior and the presence of residual DNA from dead cells. In general, these studies help to understand the persistence of antagonists when applied under postharvest conditions and will lead to optimization of time and mode of application.

1. Introduction

Postharvest green mould, caused by *Penicllium digitatum* (Per: Fr) Sacc, and blue mould, caused by *Penicillium italicum* Wehmer, are among the most economically important postharvest diseases of citrus worldwide (Talibi et al., 2014). Although, the use of synthetic chemical fungicides remains a primary method of controlling postharvest diseases, the global trend is shifting toward reduced use of fungicides on produce and hence there is a strong public and scientific desire to seek safer and eco-friendly alternatives to reduce postharvest losses (Mari et al., 2007). Biological control using microbial antagonists such as bacteria and yeasts has emerged as one of the most promising of these alternatives (Droby et al., 2009). The potential antagonist activity of many biocontrol agents (BCAs) for controlling postharvest diseases of fruit has been clearly demonstrated (Teixidó et al., 2011). In this context, the BCA *Pantoea agglomerans* strain CPA-2 has been demonstrated to be effective against the main postharvest diseases affecting citrus fruit (Teixidó et al., 2001; Plaza et al., 2004; Torres et al., 2007; Usall et al., 2008). At present, the formulated product, is now available although, it is not yet commercialized (Torres et al., 2014).

The main goal of the development and implementation of a biocontrol product is to improve the ability of the BCA to successfully control postharvest diseases under a wider array conditions and with minimal variability (Droby et al., 2003). An effective decay control depends on the ability of an antagonist to colonize the surface of fruit in both field and storage conditions and also to persist for as long as possible is vitally important. In addition, for its commercialization, the registration of a biocontrol product is required before any commercial use, as a food safety product. For EU member states, the specific requirements for registering a BCA can be found in regulation 1107/2009, which updates the existing regulations and replaces directive 91/414/EEC. Some requirements of the directive concerns fate and behavior of the microorganism in the environment and its impact on non-target species, and the residues. To address the fate of an introduced microorganism in the environment the most novel

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approach consists in designing a SCAR marker (specific-characterized-amplified-region) that will enable the natural organism to be traced among other strains of the same species in the environment (Alabouvette and Cordier, 2011). Strain-specific SCAR markers have been developed for some BCAs applied postharvest (Schena et al., 2002; De Clercq et al., 2003; El Hamouchi et al., 2008) including *P. agglomerans* strain CPA-2 (Nunes et al., 2008).

Although, conventional PCR using SCAR markers has become an attractive tool for the detection of specific microorganisms in microbial systems, this technique does not allow accurate quantification of DNA. This shortcoming has been overcome by the emergence of new techniques that can quantify nucleic acids *in vitro*. Real-time quantitative PCR (qPCR) is one of these techniques. qPCR is a sensitive and automated high-throughput technique that allows specific detection and quantification of BCAs (Larena et al., 2005; Massart et al., 2005; Pujol et al., 2006; Spotts et al., 2009; Edel-Hermann et al., 2011; Braun-Kiewnick et al., 2012b). In addition, recently it has been demonstrated that qPCR can be used to distinguish between DNA from dead and live cells by including a pretreatment of the sample with a DNA intercalating reagent, *e.g.*, propidium monoazide (PMA) (Nocker et al., 2006). This procedure is based on the integrity of bacterial cells since PMA penetrates only into compromised membrane cells (Nocker and Camper, 2009). Sample pretreatment with PMA combined with qPCR (PMA–qPCR) has been successfully tested for selective detection and quantification of foodborne pathogens (Chen et al., 2011; Elizaquível et al., 2012; Dinu and Bach, 2013) and other relevant microorganisms in the food industry (Andorra et al., 2010; Crespo-Sempere et al., 2013), but not for BCAs. In previous work, the ideal conditions for differentiating viable CPA-2 in cell suspensions and on a matrix of orange peel have been assayed (Soto-Muñoz et al., 2014a).

In this work, we used qPCR combined with PMA to quantify the populations of *P. agglomerans* CPA-2 applied on oranges in postharvest conditions. In addition, we compared these results with those obtained by dilution plating and qPCR. We also monitored the persistence and dispersion of CPA-2 in different environments such as packing lines, storage chambers, and working clothes.

2. Material and methods

2.1. Fruit

Oranges cv. Valencia Late were used in all experiments. Fruit was obtained immediately after harvest from a commercial orchard located in the Montsià area, South of Tarragona (Catalonia, Spain). Fruit was selected by hand for uniformity of size from trees without any treatment after harvest. The fruit was stored without any chemical postharvest treatment at 4 °C and 85% RH before use.

2.2. Antagonist

The CPA-2 strain of *P. agglomerans* used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain). This strain was isolated from 'Golden Delicious' apples and is currently deposited at the spanish collection of type culture (CECT, University of Valencia, Valencia, Spain), as CECT-4920. Stock cultures were stored long-term at −80 °C in cryogenic vials and subcultured on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1}dextrose, and 20 g L^{-1} agar). The activated culture was maintained on NYDA at 30 °C for 24 h and transferred to potassium phosphate buffer (pH 6.5) (0.2 M KH_2PO_4, 70 mL; 0.2 M K_2HPO_4, 30 mL, and deionised water, 300 mL) to obtain a cell suspension. This suspension was used as an inoculum for biomass production in a fermentation system. Osmotically adapted cells grown in liquid medium (5 g L^{-1} yeast extract, 10 g L^{-1} sucrose, and 25 g L^{-1} NaCl), as previously described by Cañamás et al. (2008b), were used in all studies. An appropriate volume of inoculum was added to 5 L of the liquid medium adjusted to 1×10^6 CFU mL^{-1}. Cultures were grown in a 5 L bench-top BIOSTAT-A fermenter (Braun Biotech International, Melsungen, Germany) at 30 °C with 300 rpm agitation and 100 L h^{-1} aeration. Cultures were harvested at the stationary phase (24 h) by centrifugation at 9820 g for 10 min at 15 °C in an Avanti™ J-25 centrifuge (Beckman, Palo Alto, Ca, USA). Cell pellets were resuspended in deionised water containing 10% sucrose, frozen at −20 °C overnight and freeze-dried (Cryodos, Telstar SA, Terrasa, Catalonia, Spain) at 1 Pa and −45 °C for 24 h as described by Costa et al. (2000). These lyophilised cells were packaged in a white high-density polyethylene bottle at non-vacuum atmosphere (Torres et al., 2014). For the postharvest application of the biocontrol agent, cell pellets were resuspended in 1% non-fat skim milk (Sveltesse, Nestle, Vevey, Switzeerland) and used immediately to treat oranges.

2.3. Semi-commercial trial using P. agglomerans CPA-2

A semi-commercial trial using a packing line was conducted at the IRTA-Centre in Lleida. Fruit was sprayed with the formulated and packed product of *P. agglomerans* CPA-2 previously described and adjusted to a concentration of 2×10^9 CFU mL^{-1} following standard industrial procedures in the packing line. Untreated fruit was used as controls. After treatment, oranges were placed in ten plastic boxes containing 60 fruit each. Fruit boxes were separated into two sets: one set was stored at 20 °C and 80% RH for 9 days, and the other set at 4 °C and 85% RH for 30 days.

2.4. Quantification of P. agglomerans CPA-2 on the orange surface

The quantification of *P. agglomerans* CPA-2 on oranges stored at 20 °C and 4 °C was carried out by different methods: (i) dilution plating to obtain the active-culturable population, (ii) PMA-qPCR to obtain viable cell counts, and (iii) qPCR to obtain the total cell count. The recovery of CPA-2 cells from orange surfaces was performed according to the method described by Torres et al. (2012), with minor modifications. Briefly, eight pieces of peel (16 mm diameter) were removed from each fruit. Peel samples were mixed with 20 mL of 0.05 M phosphate buffer solution in a sterile stomacher filter bag and mixed in a stomacher 400 set at normal speed for 90 s resulting mixture was filtered through a 11 μm porous filter (Whatman® International, Maidstone England). Five fruits constituted a single replicate, and four biological replicates were performed. For quantification by dilution plating, ten-fold dilutions were made in phosphate buffer solution and plated on NYDA media. Each suspension was analyzed twice. The total number of CFU was counted after incubation of plates at 30 °C for 24 h. For analysis by PMA–qPCR and qPCR, 8 mL of each suspension sample was split in two equivalents samples. One of them was used for PMA treatment as described above and the other one was used for DNA extraction as described in Section 2.4.2. The amount of resulting CPA-2 cells was calculated according to the generated standard curve by qPCR described in Section 2.4.2.

2.4.1. PMA treatment

Treatment of samples with PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride; Biotum, Hayward, USA) was performed based on optimization previously described by Soto-Muñoz et al. (2014a). Briefly, 4 mL of the sample suspension was treated with 30 μM PMA, 20 min of dark incubation and 30 min of LED light exposure. Afterwards photo-induced cross-linking, cells were centrifuged at 9727 g for