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Environmental stress responses of the *Bacillus amyloliquefaciens* CPA-8-formulated products on nectarines and peaches

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ABSTRACT

The biocontrol agent *Bacillus amyloliquefaciens* CPA-8 has been suggested as an effective alternative to chemical applications against brown rot caused by *Monilinia* spp. This study aimed to describe the population dynamics of CPA-8 on the surface of nectarines and peaches after being exposed to unfavourable environmental conditions. Two CPA-8-formulated products were obtained by fluid-bed spray-drying and then applied on fruit. Although both products included 20% sucrose plus 10% skimmed milk as protecting agents, they differ in the carrier material used during the formulation process: maltodextrin (CPA-8-formulated product called BA3) or potato starch (CPA-8-formulated product called BA4). CPA-8 has demonstrated wide tolerance to different factors such as, temperature, relative humidity and simulated rainfall. The minimal antagonist population obtained after exposure was generally higher than 10^4 CFU cm^{-2} of fruit surface, which ensures high treatment coverture and therefore, efficacy. The results also indicated that peaches were, in general, more suitable for the CPA-8 survival than nectarines. Moreover, the properties of the two CPA-8-formulated products influenced the population dynamics of the bacterium, suggesting that the BA4 CPA-8-formulated product provided higher degree of ecological fitness of CPA-8 over the fruit than the BA3 CPA-8-formulated product. The data obtained in this work led us to conclude that the integration of these CPA-8-formulated products into cropping systems is a promising strategy to achieve higher levels of brown rot control and hence contribute to a successful handling of postharvest diseases in stone fruit.

1. Introduction

Controlling fruit decays with ecologically friendly techniques is becoming popular. Using microbial antagonists has been proposed in the last decades an effective alternative to reduce or replace the chemicals applied for control of pre- and postharvest diseases (Droby et al., 2016). The biological control is safe for the appearance of fungicide-resistant population of pathogens and also for the possibility of involve toxicological risks for consumers' health. The efficacy of the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly known as *Bacillus subtilis* (Gotor-Vila et al., 2016), has been previously described against brown rot caused by *Monilinia* spp. (Casals et al., 2012; Yánez-Mendizábal et al., 2011), the wound-invading fungus that causes economically important loses (reaching even as high as 80%) of stone fruit worldwide (Mari et al., 2016; Usall et al., 2015). The key mode of action of CPA-8 is based on fengycin-like lipopeptides production (Yánez-Mendizábal et al., 2012) and the emission of effective volatile organic compounds (Gotor-Vila et al., 2017a). Moreover, data regarding registration purposes, such as molecular marker design (Gotor-Vila et al., 2016) and safety tests (Gotor-Vila et al., 2017b), have been recorded. However, while an abundance of effective beneficial microorganisms has been widely studied to control postharvest diseases, few microorganism-based products are already available in the market (Glare et al., 2012).

The main goal for developing a commercial microorganism-formulated product is to obtain large quantities of the microorganism that ensures a reasonable shelf-life (preferentially stored at room temperatures for at least twelve months) and maintains efficacy compared to fresh cells on a wide range of hosts (Droby et al., 2016; Teixidó et al., 2011). Recently, two shelf-stable and efficacious CPA-8-formulated products have been developed in a powder state by fluid-bed spray-drying (Gotor-Vila et al., 2017d). While both products contained 20% sucrose plus 10% skimmed milk as protecting agents, they mainly differ in the carrier material (maltodextrin or potato starch) used during the formulation process. However, to guaranty the biocontrol efficacy of such BCA-based products under field conditions, the technical application thresholds have to be determined.

An antagonist applied in the field presents a number of difficulties

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because BCAs would have to withstand exposure to variable and frequently hostile environmental conditions for long periods of time (Cañamás et al., 2008; Köhl and Fokkema, 1998; Lahlali et al., 2008).

Additives are often incorporated during mass production, formulation and storage or added later to spray tank mixes in order to enhance the activity of the microorganism at the target side (Andrews, 1992; Burges, 1998; Sui et al., 2015). Moreover, another way of improving survival rates for microorganisms is by understanding microbial stress response mechanisms and using this knowledge to improve resistance to unfavourable environmental conditions (Cañamás et al., 2008). Teixidó et al. (2006) demonstrated that modifying water potential in the culture medium can result in cells with improved tolerance to desiccation.

Few studies have evaluated the effect of abiotic factors interfering with the survival of BCAs, such as temperature, relative humidity (RH) or UV radiation (Calvo-Garrido et al., 2014a; Cañamás et al., 2008; Lahlali et al., 2011). In the orchard, microbial populations are subjected to daily fluctuations of the mentioned factors that could be controlled during postharvest storage (Calvo-Garrido et al., 2014a; Magan, 2001; Teixidó et al., 2010). However, the effect of other weather phenomena such as rainfall events on BCAs has been barely studied. Calvo-Garrido et al. (2014b) specifically evaluated the population dynamics of the yeast *Candida sake* exposed to simulated rainfall with different rain intensities, rain volumes and time length between rain events. These factors have been described for influencing wash-off of agrochemicals on different types of crop plants (Fife and Nokes, 2002; Hunsche et al., 2007).

Effective colonisation, high population and viability of BCAs on fruit surfaces have been considered important aspects in the successful control of postharvest diseases. If appropriate environmental conditions are not consistently available, BCA populations may fail to reduce disease incidence and severity, and may not recover as rapidly as pathogen populations when conducive conditions occur (Garrett et al., 2006). The environmental conditions mentioned directly influence the capability for growth and establishment of BCAs on the fruit surface. Thus, it is important to identify the environmental niche in which an individual BCA can actively grow as this enables abiotic threshold criteria and hence design application programs to achieve high treatment efficacy (Teixidó et al., 1998).

The aim of this work was to assess the persistence of the BCA *B. amyloliquefaciens* CPA-8 on the surface of nectarines and peaches after being treated with two CPA-8-formulated products and exposed to different environmental conditions. In order to do this, we studied the main factors which could affect the CPA-8 survival under field conditions: (i) temperature, (ii) relative humidity and (iii) wash-off caused by simulated rainfall.

2. Materials and methods

2.1. Microorganism and culture conditions

B. amyloliquefaciens CPA-8 was isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were 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) at 30 °C for 24 h when required.

Fresh bacteria cultured overnight at 30 °C in NYDA plates and suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1} ; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6.5) were used to prepare an appropriate volume of inoculum to inoculate a 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) and 5 L (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) laboratory scale bioreactors containing growth medium previously described by Yánez-Mendizábal et al. (2012a) and optimised by Gotor-Vila et al. (2017d). The initial concentration was adjusted at 10^6 CFU mL^{-1}. CPA-8 cells were grown for 68–72 h at 30 °C to obtain high endospore concentration (Gotor-Vila et al., 2017d). Agitation was set to 300 rev min^{-1}, the air feeding rate was 0.33 vvm and antifoam (1 mL per litre) was added if needed (30% Simethicone emulsion USP, Dow Corning®, USA).

2.2. CPA-8-formulated products

CPA-8 cells were harvested by centrifugation at 9820*g* for 12 min at 10 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended approximately at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process (Yánez-Mendizábal et al., 2012b). Two CPA-8 formulated products were obtained by using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) according to the protocol developed by Gotor-Vila et al. (2017c) and by Gotor-Vila et al. (2017d). Briefly, CPA-8 cells were mixed with the protective substances 20% sucrose plus 10% skimmed milk and then fluid-bed spray-dried with 300 g of powdered carrier material previously loaded into the drying camera. Two different carriers were used: maltodextrin (CPA-8-formulated product called 'BA3') and potato starch (CPA-8-formulated product called 'BA4').

2.3. Persistence of CPA-8-formulated products on nectarines and peaches under different environmental conditions

2.3.1. Treatment of fruit with the CPA-8-formulated products

For each treatment, condition and sampling time assessed, 20 fruits were randomly selected without visible injuries and rots and as much as homogeneous in maturity and size. Each setup consisted of four replicates with five fruits each. CPA-8 suspensions in water were prepared from the CPA-8-formulated products BA3 or BA4 and adjusted at 10^7 CFU mL^{-1}. The suspensions were sprayed on nectarines and peaches until run off by using a manual backsprayer (ARPI 18 L, CA Bovi, Lleida, Catalonia, Spain) and then air-dried for 2 h at room temperature. The spray system was kept close to the fruit to reduce bacterial movement by aerosol from the application side.

2.3.2. Effect of temperature

The effect of two temperatures of storage (0 and 20 °C) on CPA-8 populations was evaluated on 'Rome Star' peaches and 'Big Top' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition and sampling time were placed on packing trays and stored in two climatic chambers programmed at the temperatures mentioned. The population dynamics of CPA-8 was assessed after 0, 1, 2, 5 and 7 days and after 0, 2, 7, 15, 30 and 60 days in fruit stored at 20 and 0 °C, respectively.

2.3.3. Effect of RH

The effect of three different values of RH (40, 60 and 85%) on CPA-8 populations was evaluated on 'Gladys' peaches and 'Fantasia' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition and sampling time were placed on packing trays, covered with plastic chambers, sealed, and stored in climatic chambers programed at 20 °C. The distinct RH values inside the plastic chambers were achieved by placing a dehumidifier (FDC32S, FRAL, Carmignano di Br., PD, Italy), and monitoring RH during storage with an external data logger (Testo 175H1, Testo Inc., Sparta Township, NJ, USA). The population dynamics of CPA-8 cells under the mentioned storage conditions was assessed after 0, 1, 2, 3, 6 and 9 days on peaches and after 0, 1, 2, 3, 8 and 10 days on nectarines.

2.3.4. Effect of simulated rainfall

Two different trials were conducted in order to evaluate the