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Combined effect of antagonistic yeast and modified atmosphere to control *Penicillium expansum* infection in sweet cherries cv. Ambrunés

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ABSTRACT

Fruit decay caused by pathogenic moulds is a major concern in the postharvest quality and shelf life of fruit. Blue mould decay is caused by *Penicillium expansum* (*P. expansum*) and is one of the most important postharvest diseases in cherries (*Prunus avium* L.). Synthetic fungicides are the main medium used to control pathogenic moulds. However, alternative approaches are available for developing safer technologies to control postharvest disease. An integrated approach that combines biological control, using antagonistic yeasts and modified atmosphere packaging (MAP) with cold storage is a promising alternative to synthetic fungicide treatment. In this work, two microperforated films (M10 and M50) and two antagonistic yeast strains (*Hanseniaspora opuntiae* L479 and *Metschnikowia pulcherrima* L672) were evaluated for their effectiveness to control the development of *P. expansum* in wounded cherries stored at 1 °C. Results showed that the microperforated films had fungistatic effects, particularly M50, due to the level of CO_2 achieved (mean CO_2 of 11.2 kPa at 35 days), and the decrease in disease severity. Antagonistic yeasts, particularly *Metschnikowia pulcherrima* L672, delayed the development of *P. expansum* and decreased disease incidence and severity. The combination of MAP and antagonistic yeasts was the most effective approach to control *P. expansum*, during cold storage.

1. Introduction

The control of postharvest fruit pathogens is one of the most important challenges within the fruit industry. The proliferation of moulds, which, in addition to causing substantial economic losses, may also have serious health implications due to contamination of the fruit by mycotoxins (Drusch and Ragab, 2003).

Synthetic fungicides remain the most effective tool to control postharvest fungal diseases in perishable fruit and vegetables. However, the use of synthetic fungicides is under severe legal control due to acute, cumulative, and synergistic effects (Tripathi and Dubey, 2004). In 2014, the European Food Safety Authority (EFSA) found detectable residues of synthetic compounds in approximately 45% of the tested food samples, with 1.9% of all the samples exceeding the legal limit. Moreover, pesticides not approved in the European Union were detected (EFSA, 2014).

The need to decrease the use of synthetic fungicides, concerns about health and environmental implications, and the development of fungicide-tolerant pathogenic strains (Ma and Michailides, 2005) has motivated the development of alternative approaches to control plant diseases. Indeed, several physical and biological methods have demonstrated their potential (Tripathi and Dubey, 2004). One tool that has been used to extend the postharvest quality of sweet cherry is modified atmosphere packaging (MAP) (Remón et al., 2000; Serrano et al., 2005). MAP involves the use of permeable films to create a modified atmosphere that is low in O_2 and high in CO_2. This decreases the respiration rate of the fruit and extends its shelf life (Jacxsens et al., 2002; Rodríguez and Zoffoli, 2016). Successful MAP must maintain near optimum levels of O_2 and CO_2 to achieve the beneficial effects. However, the optimum levels of O_2 and CO_2 (2–10% of O_2 and 5–20% of CO_2) for sweet cherry depend on cultivar and ripening stage (Serrano et al., 2005).

Biological control using microbial antagonists has also emerged as a promising technique to control postharvest pathogens and decrease synthetic fungicide use (Sharma et al., 2009). The selection and application of antagonists are primarily focused on yeasts because they have many of the characteristics desired in an ideal antagonistic agent. This includes their ability to rapidly colonise and grow in surface wounds and subsequently out-compete pathogens for nutrients and space (Wilson and Wisniewski, 1989). The antagonistic yeasts, *Hanseniaspora opuntiae* L479 (HO-L479) and *Metschnikowia pulcherrima* L672 (MP-L672), isolated from the common fig (*Ficus carica* L.), are promising

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candidates, which have shown the ability to control fruit pathogens *in vitro* (Ruiz-Moyano et al., 2016).

The main barrier concerning the industrial application of antagonists as a postharvest treatment strategy is the lack of efficacy and consistency of this method when used alone under commercial conditions (Droby et al., 2001). However, the convenience and effectiveness of combining biological methods with other alternate treatments to improve control of fruit pathogens have been demonstrated (Droby et al., 2002; Zheng et al., 2007). Moreover, level the effectiveness of the combined treatments was often comparable to synthetic fungicides (Droby et al., 2002; Zheng et al., 2007). Yet, a limited number of studies have addressed the suitability of combined treatments.

Decay by *Penicillium* sp., particularly the blue mould *Penicillium expansum* (PE-M639), is a major concern in the sweet cherry industry (Serradilla et al., 2013; Serrano et al., 2005; Venturini et al., 2002). Therefore, this work aimed to control blue mould (PE-M639) in sweet cherries (*Prunus avium* L. cv. Ambrunés) by using an integrated approach involving MAP and two antagonistic yeasts, HO-L479 and MP-L672, with chilled storage.

2. Material and methods

2.1. Plant material

'Ambrunés' sweet cherries (*P. avium* L.) were harvested from 14-year-old sweet cherry trees of the 'Ambrunés' cultivar, on *P. avium* L. rootstock from an experimental orchard, located 400 m above sea level in Cabrero (lat. 40° 06′ 40″ N, long. 5° 53′ 20″ W), in the "Valle del Jerte" (Cáceres, Spain). The cherries were harvested at random from multiple trees and transported to the distribution centre within 1 h. The fruit was then sorted on the basis of ripening stage, as Serradilla et al. (2013). Cherries at ripening stage 2, corresponding at commercial ripening stage for this cultivar, were selected for this study.

2.2. Microbial preparation

Two yeast strains, *H. opuntiae* L479 (HO-L479) and *M. pulcherrima* L672 (MP-L672), isolated from breva crops (Villalobos et al., 2014), were chosen based on their antagonistic capability *in vitro* (Ruiz-Moyano et al., 2016). The yeast strains were cultivated in nutrient yeast dextrose broth (NYDB) on a rotary shaker at 120 rpm, at 20 °C for 48 h. Cells were collected by centrifugation, 16,000*g* for 10 min and washed twice with phosphate buffer (pH = 7). The final pellets were resuspended in sterile distilled water to 10^8 cells/mL, using a Neubauer chamber.

Penicillium expansum M639 (PE-M639) (Ruiz-Moyano et al., 2016) mould was grown on potato dextrose agar (PDA) at 25 °C for 10 days. The spores were collected in distilled water with 0.05% (v/v) Tween 80 (Scharlau, Spain). The final pellets were resuspended to 10^5 spores/mL in sterile distilled water, using a Neubauer chamber.

2.3. Inoculation of microorganism and MAP storage

'Ambrunés' sweet cherries were hydrocooled with water containing 100 μg L^{-1} of sodium hypochlorite, at 1–4 °C for 3 min. The cherries were then rinsed by immersion in sterile water for 3 min, completely air-dried at room temperature, then deliberately wounded (3 mm wide × 3 mm deep; one wound per fruit) with a sterilised cork borer. Each wound was then inoculated by using one of four combinations (Table 1):

a) Cell suspension (20 μL) of HO-L479, followed by 20 μL of spore suspension (PE-M639).
b) Cell suspension (20 μL) of MP-L672, followed by 20 μL of spore suspension (PE-M639).
c) Sterile water (20 μL) followed by 20 μL of spore suspension (PE-M639).
d) Sterile water alone (40 μL), as a control.

The inoculated cherries were packaged in transparent polyethylene punnets (24 × 14 cm, 1180 cm^3), with thirty fruit per punnet, and sealed with one of three 40-μm thick biaxially oriented polypropylene (BOPP) films (ACSA Films, Valencia, Spain) under atmospheric conditions, obtaining a total of 12 batches (Table 1). The films used were: macroperforated film (Macro), with six holes (∅ = 9 mm); microperforated BOPP film with one hole per 10 mm (a total of 16 holes, ∅ = 100 μm) (M10); and microperforated BOPP film with one hole per 50 mm (a total of three holes, ∅ = 100 μm) (M50). All punnets were stored at 1 °C and 90–95% RH, in the dark. Three punnets of each batch were randomly sampled at 0, 7, 14, 21, 28 and 35 days of storage. The experiment was repeated twice.

Table 1
Modified atmosphere packaged sweet cherries (*Prunus avium* L. cv. Ambrunés) batches stored at 1 °C for 35 days, including film type and inoculum.

Batch	Film	Inoculum	Number of punnets
1	Macro	Control	15
2	Macro	PE-M639[a]	15
3	Macro	HO-L479[b] + PE-M639	15
4	Macro	MP-L672[c] + PE-M639	15
5	M10	Control	15
6	M10	PE-M639	15
7	M10	HO-L479 + PE-M639	15
8	M10	MP-L672 + PE-M639	15
9	M50	Control	15
10	M50	PE-M639	15
11	M50	HO-L479 + PE-M639	15
12	M50	MP-L672 + PE-M639	15
Total			180

[a] *Penicillium expansum* M639.
[b] *Hanseniaspora opuntiae* L479.
[c] *Metschnikowia pulcherrima* L672.

2.4. Analysis of headspace gas composition of the headspace

The evolution of CO_2 and O_2 levels in the headspace of the packages were analysed by a Checkmate 3 headspace gas analyser (PBI Dansensor, Denmark) between 7 and 35 days of storage. The gas analyser needle was inserted through a silicone rubber seal attached to the outside of the film. The results were expressed as kPa CO_2 and kPa O_2.

2.5. Dynamics of yeast and mould counts in the wounds

The evolution of the inoculated yeast and mould strains was monitored by enumeration on solid cultures. The inoculated area of five fruit per punnet was obtained with a sterile bisturi and homogenised in nine volumes of sterile 0.1% (p/v) peptone water in a stomacher (Lab Blender, Model 4001, Seward Medical, London, UK) for 60 s. Then, serial decimal dilutions in peptone water were performed and 0.1 mL aliquots were plated on rose bengal agar (Oxoid), supplemented with chloramphenicol (RBCA) and PDA, pH 3.5, adjusted with tartaric acid. Plates were incubated at 25 ± 1 °C for 4 days, and results were expressed as log CFU/g. Analyses were performed in triplicate at 0, 7, 14, 21, 28 and 35 days of storage.

2.6. Control of Penicillium expansum disease

The incidence and severity of PE-M639 disease were recorded at 7, 14, 21, 28 and 35 days of storage. All fruit contained in three punnets per batch was measured per day of sampling. The disease incidence was expressed as a percentage of fruit with rot symptoms. The disease severity was expressed as mm of injured tissue. The disease severity