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Volatile organic compounds produced by Antarctic strains of *Candida sake* play a role in the control of postharvest pathogens of apples

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HIGHLIGHTS

- Psychrotrophic yeasts isolated from Antarctica were used as biocontrol agents.
- VOCs produced by *Candida sake* strains inhibited apple pathogens.
- The VOCs produced by *C. sake* 41E were identified using SPME GC–MS.

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ABSTRACT

In this study the strategy of isolating psychrotrophic, non-pectinolytic yeasts able to grow in apple juice as potential biocontrol agents was a successful approach. Thirty-four yeasts isolated from Antarctic were able to maintain rot incidence caused by *P. expansum* and *B. cinerea* under 25% on apples stored at 0–1 °C. Two of the isolates, identified as *Candida sake*, produced antifungal volatile organic compounds (VOCs) which inhibited the growth of five pathogens of apple (*P. expansum, B. cinerea, A. alternata, A. tenuissima,* and *A. arborescens*). This is the first report of VOCs produced by *C. sake*, as well as the first study of the inhibitory activity of VOCs produced by yeasts against species of *Alternaria* that cause postharvest apple rot.

In vitro studies were performed on Apple Juice Agar at 0 °C and 25 °C due to the importance of evaluating antifungal activity in similar conditions to where the biocontrol agent are intended to be used. The VOCs produced by *Candida sake* strain 41E were also effective on *in vivo* assays to control *P. expansum* in Red Delicious apples.

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1. Introduction

In the southern hemisphere, apples are harvested from February to April. In order to provide fruit throughout the whole year, maintain quality and reduce spoilage, apples are stored at low temperature (0–1 °C). The development of fungal rots, mainly by *Penicillium expansum* (Romano et al., 1983), however, cannot be avoided. Other fungal pathogens such as *Botrytis cinerea* (Rosenberger, 1990) and *Alternaria* spp. (*A. alternata*, *A. tenuissima,* and *A. arborescens*) (Serdani et al., 2002; Kou et al., 2014; Jurick II et al., 2014) have also been reported to cause fruit decays in cold storage. The application of postharvest fungicides to apples prior to being placed in storage is the main management strategy used to decrease fruit losses due to decay. In Uruguay, integrated production guidelines (Scatoni et al., 2005) recommend the use of three synthetic fungicides (iprodione, imazalil, and captan) to control postharvest diseases of apples when fruit is to be stored for more than 3 months. Due to consumer demands to reduce chemical residues in food and concern about the environment, there has been great interest in developing alternative control methods. In this regard, biological control has received considerable attention as a potential alternative (Wisniewski et al., 2016; Droby et al., 2016; Vero et al., 2009).

In order to prevent the development of fungal pathogens on apples during cold storage a biocontrol agent must be able to grow and colonize fruits at low temperatures. Thus, the strategy of isolating psychrotrophic microorganisms from cold environments and evaluating them for their biocontrol potential is a logical approach. Other characteristics, such as the ability to readily

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colonize apple wounds, which are the main infection court of necrotrophic, decay pathogens, and the inability of the biocontrol agent to produce pectinolytic enzymes that could degrade apple tissue should be taken into account in the selection of an antagonist. Other mechanisms, including nutrient competition, the production of soluble or volatile inhibitory metabolites, direct hyphal parasitism, and the induction of host resistance have also been demonstrated to play a meaningful role in the biocontrol of postharvest pathogens by microbial antagonists (Wisniewski et al., 1991; Vero et al., 2002; Droby et al., 2002; Mohamed & Saad, 2009; Tongsri and Sangchote, 2009; Huang et al., 2011; Romanazzi et al., 2016; Spadaro and Droby, 2016; Liu et al., 2017).

Among the various modes of action of postharvest biocontrol agents, the production of antifungal volatile organic compounds (VOCs) by postharvest biocontrol agents (Batista Fialho et al., 2010; Huang et al., 2012, 2011; Di Francesco et al., 2015; Mari et al., 2016) has been receiving increased attention. VOC-producing microorganisms have the potential to be used as biofumigants direct or their active constituents can be used independently. The use of VOC-producing microbes thus represents an attractive and logical strategy as an alternative approach for the control of postharvest diseases of produce, especially since commodities are stored in closed containers during storage and shipment. VOCs are low molecular weight, carbon-containing compounds with a low polarity, and a high vapor pressure (Vespermann et al., 2007; Bennett et al., 2012). They are produced by a wide range of microorganisms, including bacteria, molds and yeasts. Some VOCs have antimicrobial activity and some are involved in microbial interactions, acting as signaling and quorum sensing compounds (Bennett et al., 2012). Kanchiswamy et al. (2015) reported that there is evidence to suggest that microbial VOCs are ecofriendly and thus could be used in agricultural practices to prevent fungal pathogen development. The composition of the mixture of antifungal volatiles produced by a biocontrol agent needs to be comprehensively analyzed in order to ensure that they do not cause any known or perceived threats to humans, and the environment in general.

The present study focused on the identification and evaluation of psychrotrophic yeasts, isolated from water and soil samples from King George Island in the sub-Antarctic region, for their potential use as postharvest biocontrol agents for the control of postharvest diseases of apple stored at low temperature. In the course of the evaluation, the production of antifungal VOCs of all the selected isolates was examined. Additionally, the composition of VOCs were also evaluated.

2. Materials and methods

2.1. Pathogens, biocontrol strains and biocontrol assay

Native strains of *Penicillium expansum* and *Botrytis cinerea*, previously isolated and identified from rotting apples (Pianzzola et al., 2004; Gepp et al., 2012) were used in the current study, this strains were kept in agar slants covered with mineral oil at 4 °C. Additionally, isolates of *Alternaria alternata* CBS916.96, *Alternaria tenuissima* CBS 124.277, and *Alternaria arborescens* CBS 102.605 were obtained from the Centraal Bureauvoor Schimmel cultures (CBS, Wageningen, The Netherlands) and used in the present study.

2.1.1. Origin and selection of potential biocontrol agents

Sixty-one yeast strains isolated from soil and water samples collected in King George Island, South Shetland Islands, Antarctica, (62°02′558°21′W) in 2012 and 2013 were used in this study (Martínez et al., 2016). All strains were evaluated for their ability to produce pectinolytic enzymes using the method described by Merín et al. (2011) with modifications. Briefly, Yeast Nitrogen Base (YNB) (Difco, Detroit, MI, USA) supplemented with 1% pectin as the only carbon source was used to detect enzymatic activity. Each strain was point inoculated on the medium and incubated at 25 °C for 48–72 h. Enzymatic activity was indicated by the formation of a clear halo around the colonies against a purple background after flooding the plate with Lugol's solution. Strains that produced pectinases were not subjected to any further evaluation. The non-pectinase-producing strains were then evaluated for their ability to grow in apple juice at low temperature (0 °C). Sterile apple juice was inoculated with each yeast suspension to reach a final concentration of 1×10^4 cell/mL and incubated at 0 °C for 4 days. Microbial growth was evaluated daily by measuring optical density (OD) at 600 nm.

2.1.2. Biocontrol assay

The selected yeast strains that were negative for pectinolytic activity and positive for growth at 0 °C were further evaluated for their biocontrol ability against *Penicillium expansum* and *Botrytis cinerea* in wounded 'Red Delicious' apples stored at 0–1 °C. Apples were first surface-disinfected with 70% ethanol. After drying, five wounds (4 mm deep × 2 mm wide) were made along the equator of each apple. Each wound was then inoculated with 10 μL of a yeast suspension made from a two-day-old PDA culture, that had been pelleted and added to sterile water to obtain a final concentration of 1×10^7 cell/mL. Cell concentrations were determined, adjusted, and confirmed by microscopic count in a Neubauer chamber. Control wounds were inoculated with 10 μL of sterile water. Wounds were allowed to dry at room temperature for two hours and then subsequently inoculated with 10 μL of a 1×10^4 conidia/mL conidial suspension of either *B. cinerea* or *P. expansum*. Six apples were used per treatment (n = 30). Apples were stored in a cold chamber at 0–1 °C for 3 months after which disease incidence (measured as the percentage of infected wounds) was evaluated. The statistical analysis of disease incidence was performed using a generalized linear model, assuming a binomial distribution, with a logit transformation (maximum likelihood). Treatments were compared by the DGC test (Di Rienzo et al., 2002) at a significance level of 0.05 using the InfoStat software package (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, 2009).

2.2. Production of antifungal volatile compounds

Production of antifungal volatile compounds was determined as described by Huang et al. (2011) with some modifications. The center of petri plates (5 cm diameter) containing 5 mL of Apple Juice Agar (sterile apple juice at pH = 5 with 2% agar) (AJA) were inoculated with a mycelial agar plug (5 mm diameter) removed from a non-sporulating, one-day-old AJA culture of *P. expansum*. At the same time, another petri plate containing 5 mL of AJA was surface inoculated with 50 μL of a yeast suspension of 1×10^7 cell/mL, evenly spread across the surface of the medium with a sterilized glass spreader. The lids of both petri plates (pathogen and yeast inoculated) were removed. The double dish sets (DDS) were joined and sealed using double layers of Parafilm® to make closed chambers. Negative controls consisted of DDSs prepared from plates inoculated with *P. expansum* covered with uninoculated AJA plates. All DDSs were incubated at 25 °C for 3 days. Three replicates for each treatment were used (n = 3). The pathogen colony diameter was measured after the incubation period. The percentage of inhibition of mycelial growth of *P. expansum* by the volatile compounds produced by the yeasts was calculated on the basis of the difference with the fungal colony diameter in control plates.

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