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The detection of fungal diseases in the 'Golden Smoothee' apple and 'Blanquilla' pear based on the volatile profile

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ABSTRACT

Blue mould caused by *Penicillium expansum* is one of the major pathogens causing serious losses during the postharvest and storage periods of apples and pears. *Rhizopus stolonifer* has also been identified as the causative agent of important rot losses in these fruit in packinghouses after humid spring seasons. An early disease detection system could help to reduce such losses. Biotic interactions affect the emission of volatile compounds during infection, and this changed the volatile profile of 'Golden Smoothee' apples and 'Blanquilla' pears inoculated with *P. expansum* and *R. stolonifer* throughout their shelf-life at 20 °C for up to 7 days. Dynamic headspace-gas chromatography was used to determine if infected fruit emitted distinct volatile compounds different from those of non-infected fruit and if volatile emissions could be detected before the infection was visible in the fruit. According to the results obtained in 'Golden Smoothee' apples inoculated with both pathogens, *Z*-3-hexenyl 2-methylbutanoate could be a potential biomarker because it was quantified before these diseases were visible and was not detected in non-inoculated control fruit. Similar results were obtained for 2-butanone and α-pinene in 'Blanquilla' pears inoculated with *R. stolonifer*.

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1. Introduction

Blue mould caused by *Penicillium expansum* Link is the most important postharvest disease of apples and pears. *Rhizopus stolonifer* (Ehrenb. Ex Fr.) Lind causes *Rhizopus* rot in pome fruit. However, while *Rhizopus* rot is a serious disease of stone fruit, it has not been considered to be a major pome fruit disease because the development of this species is inhibited at temperatures below 5 °C. Therefore, the rot is prevented at the storage temperatures recommended for storing apples and pears (Barkai-Golan, 2001). However, *Rhizopus* rot causes important rot losses in Spanish packinghouses in stored apples, and especially in pears after humid spring seasons. *R. stolonifer* strains isolated from decayed fruit at cold storage conditions could cause disease in pears and apples artificially inoculated and stored at −1 °C and 0 °C, respectively, in our laboratory (Usall et al., 2013).

Methods for controlling postharvest fungal diseases of fresh fruit vary and depend on the requirements of the target markets. Chemical fungicide treatments are the main means to control these diseases. The application of fungicide mixtures has proven effective for controlling rot in apples (Barkai-Golan, 2001) and pears (López and Riba, 1999), but fungicide residues remain in the fruit as a consequence of postharvest treatment (Villatoro et al., 2009a,b) and are not desired by consumers in some markets. Various alternative methods have been tested to replace chemicals, such as heat treatments (Dang et al., 2008), biocontrol (Teixidó et al., 2011), modifying the storage atmosphere (Akbudak et al., 2009) and low-toxicity treatments, such as peracetic acid (Alvaro et al., 2009). However, these methods are limited and raise concerns.

The detection of diseases in stored apples and pears is very difficult, especially in controlled atmosphere conditions, in which storage chambers are closed for long periods of time and disease development in the fruit cannot be visually detected. When these diseases can finally be observed, they are in a very advanced stage and interventions to reduce losses are not possible. Rotten fruit usually produce a characteristic odour that sometimes differs depending on the pathogen causal agent (Wihlborg et al., 2008) and the fruit. Several studies have been performed to evaluate the changes in the volatile emissions caused by fungal infection in apples (Vikram et al., 2004a,b; Karlshøj et al., 2007). Vikram et al. (2004a,b) reported that compounds such as dimethyl ether and propanal were specific to 'Cortland' apples

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inoculated with *P. expansum*, while fluoroethene and 3,4-dimethyl-1-hexene were specific to 'McIntosh' apples inoculated with the same pathogen. However, in all cases, these compounds were detected when visual damage had already occurred (Vikram et al., 2004a,b; Vandendriessche et al., 2012).

Convenient measures and decisions could be taken and losses diminished if pathogens could be detected early when they just start to develop in fruit. The need to develop sensitive, rapid and cost-effective methods to detect and identify pathogens is clear. With biotechnology advances, techniques have been developed to diagnose diseases, such as the enzyme-linked immunosorbent assay (ELISA) (Vandendriessche et al., 2012) and molecular diagnostic tools, such as the polymerase chain reaction and the reverse transcriptase polymerase chain reaction (Schaad et al., 2003). Although these molecular techniques are quite sensitive, samples need to be taken from boxes stored and piled in closed chambers, and both analytical techniques could not be used for the early detection of fungal infections.

Although the first comprehensive studies of identifying volatile compounds that control mould during the postharvest storage of fruit were performed approximately 16 years ago (Archbold et al., 1997), studies to detect early non-visual *P. expansum* and *R. stolonifer* infections in apples and pears via volatile compounds have not been previously published in the literature to our knowledge.

The aim of this study was to determine whether specific volatile compounds can be detected in *R. stolonifer* and *P. expansum* fungal infection before the infection spreads throughout the 'Golden Smoothee' apple and 'Blanquilla' pear fruit during a shelf-life at 20 °C or DAI. This approach could serve as a possible early detection rot system.

2. Materials and methods

2.1. Plant material and postharvest handling

Apples (*Malus domestica* L. cv. Golden Smoothee) and pears (*Pyrus communis* cv. Blanquilla) were obtained from commercial orchards in Lleida (north-eastern Spain). Immediately after harvest, four 10-kg lots of each fruit cultivar of uniform size and lacking physical injuries or apparent infections were selected. The fruit were placed in plastic trays and delivered to laboratory. Three apples and pears set were surface disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water and allowed to dry at room temperature. One set was inoculated with *R. stolonifer*, a second set was inoculated with *P. expansum* and a third set was only wounded with a nail as control fruit. The fourth set of each fruit cultivar was used for determination of fruit maturity parameters at harvest. Inoculated and wounded-control fruit were volatile analyzed after 8 h, 24 h, 48 h, 4 d and 7 d after pathogen inoculation at 20 °C (DAI).

2.2. Determination of fruit maturity parameters

The colour was measured on two opposite sides on each fruit. The measurements were made by placing the head of a portable tristimulus colorimeter (Chromameter CR-200, Minolta, Japan) at the midpoint between the stem and the calyx end and recording the chromaticity values of the fruit in the L^* a^* b^* space coordinates (McGuire, 1992). Hue angle (h°), calculated as $h^\circ = \arctan b^*/a^*$, was used to indicate the colours of apples and pears. The flesh firmness was measured at two opposite equatorial peeled sides on the equator of each fruit with an Effegi penetrometer (FT 327; Effegi, Alfonsine, Italy) equipped with an 11 mm and 7.9 mm diameter plunger tip for apples and pears, respectively. The results were expressed in newtons. The soluble solids concentration (SSC) was determined by measuring the refractive index of juice of the same fruit used to determine the firmness, and the data were expressed as a percentage (g per 100 g fresh weight (FW)). The acidity was measured as follows: 10 mL of pulp juice was diluted with 10 mL H_2O and titrated with 0.1 M NaOH solution. The acidity was expressed as grams of malic acid per litre of juice. The data on the maturity indices represent the mean of 20 individual fruit.

2.3. Pathogens

P. expansum (CMP-1) and *R. stolonifer* (CMR1) (Nunes et al., 2001a,b; Vilanova et al., 2012) were isolated from decayed apples after several months in storage and maintained on potato dextrose agar medium (PDA; 200 mL of extract from boiled potatoes, 20 g of dextrose, 20 g of agar and 800 mL of water). These pathogens are the most virulent isolates of the University of Lleida-IRTA culture collection, and they were periodically grown on wounded pome fruit and re-isolated to maintain virulence.

2.4. Pathogens fruit inoculation and incubation

Both pathogens were grown on PDA for 7–10 days at 21 °C, and a conidial suspension was prepared by adding 10 mL of sterile distilled water containing 0.01% Tween 80 to the surface of the cultures. The conidia were scraped from the agar using a sterile loop, and the suspension was subsequently discarded into a test tube. The suspension was then sonicated for 5 min to improve the conidial dispersion, and the concentration of the solution was adjusted to the desired concentration using a haemocytometer.

Eight kilograms of fruit per pathogen (2 kg per replicate × 4) were wounded with a nail by making a 2-mm diameter and 2 mm deep injury near the stem (top) and the calyx (bottom). The wounds were inoculated with 15 μL of an aqueous suspension of 1×10^4 or 5×10^3 conidia mL^{-1} of *P. expansum* or *R. stolonifer*, respectively. The fruit were dried at room temperature and then incubated in four 8 L Pyrex containers at 20 °C to analyse the volatile compounds 8 h, 24 h, 48 h, 4 d and 7 d after pathogen inoculation (DAI). The 8 kg of control fruit were only wounded with a nail as explained above.

2.5. Analysis of volatile compounds

The volatile compounds were measured as described by Altisent et al. (2011). Eight kilograms of fruit (2 kg per replicate × 4) per inoculated, as described above, and wounded-non-inoculated cultivar fruit were placed separately in four 8 L Pyrex containers through which an air stream (900 mL min^{-1}) was passed for 4 h. The resulting effluent was then passed through an ORBO-32 adsorption tube filled with 100 mg of activated charcoal (20/40 mesh), from which volatile compounds were desorbed by agitation for 40 min with 0.5 mL of diethyl ether. The volatile compounds were identified and quantified on a HP 5890 series II gas chromatograph (Hewlett-Packard Co., Barcelona, Spain) equipped with a flame ionisation detector (GC-FID) using a polyethylene glycol capillary column with cross-linked free fatty acid as the stationary phase (FFAP; 50 m × 0.2 mm × 0.33 μm). A volume of 1 μL of the extract was injected into the column for all of the analyses. The oven program was set at 70 °C (1 min), and the temperature was first raised by 3 °C min^{-1} to 142 °C and later by 5 °C min^{-1} to 225 °C and then kept constant for 10 min at this latter temperature. Helium was used as the carrier gas (42 cm s^{-1}), with a split ratio of 40:1. The injector and detector were held at 220 and 240 °C, respectively. A second capillary column (SGE, Milton Keynes, U.K.) with 5% phenyl polysilphenylene–siloxane as the stationary phase (BPX5, 30 m × 0.25 mm i.d. × 0.25 μm) was also used to identify the compounds at the same operating conditions as described above. The

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