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Detection of α -Farnesene and 6-Methyl-5-hepten-2-one Involved in the Development of Apple Superficial Scald by PTR-ToF-MS

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Abstract

To date, the accepted hypothesis about the development of superficial scald in apple fruit is related to the accumulation of harmful α -farnesene auto oxidation products, such as conjugated trienols or 6-methyl-5-hepten-2-one (MHO). The aim of this research was the implementation of an alternative, rapid, reliable, analytical technique based on PTR-ToF-MS (proton transfer reaction – time of flight – mass spectrometry) to measure the volatile organic compounds released during the progression of this disorder in an apple. This assessment was performed with three specific tissues (skin, under skin, and pulp) taken into consideration, as well as the comparison between control and treated samples with 1-MCP applied before storage. The results described here suggest the use of MHO as a novel biochemical marker to monitor the oxidative stress of apple fruit, since its concentration is significantly correlated with the early development of visible superficial scald symptoms.

INTRODUCTION

Superficial scald is a postharvest physiological disorder affecting pome fruits normally associated with chilling injury and oxidative stress occurring during storage (Watkins et al., 1995). Together with bitter pit and internal browning, superficial scald has a considerable implication in fruit marketability and quality; therefore it can be considered one of the major apple storage disorders (Mattheis, 2008). Superficial scald symptoms show necrotic areas on the surface layers of the hypodermal cortical tissue cells, resulting in a browning coloration of the fruit skin, without impacting the inner flesh tissues (Lurie and Watkins, 2012). The development of scald seems to be cultivar specific, since specific cultivars, such as 'Granny Smith', 'Red Delicious', 'Pink Lady' and 'Fuji' are more susceptible to the onset of this disorder (Whitaker et al., 2000; Tsantili et al., 2007).

The effect of superficial scald in apple is still highly discussed, since the real aetiology of this phenomenon is not yet completely elucidated (Lurie and Watkins, 2012). To date, the most investigated and accepted hypothesis about scald development is related to the accumulation of products derived by the autoxidation of α -farnesene, such as conjugated trienols (Whitaker et al., 1997; Whitaker et al., 2000; Rowan et al., 2001) or 6-methyl-5-hepten-2-one (MHO) (Mir et al., 1999; Wang and Dilley, 2000; Rudell et al., 2009) which cause serious damage to the hypodermal tissue. The resulting browning coloration in the fruit skin with the ongoing superficial scald phenomenon can be ascribed to an oxidation process of polyphenols, accumulated in the cell vacuole as secondary metabolites (Du and Bramlage, 1995; Abbasi et al., 2008).

To date, the majority of the studies focused on the basic physiology of superficial scald were focused on the analysis of α -farnesene and its autoxidation products by using GC-MS, HPLC-UV/vis-APCI-MS or spectrophotometric analysis of hexane extraction by apple skin (Lurie and Watkins, 2012). Technological improvements towards the assembling of advanced equipment and the release of data processing algorithms are fundamental requirements to employ untargeted metabolomics approaches in order to

have a more comprehensive characterization of this phenomenon. This knowledge would allow the definition of valuable biochemical markers for an on-line and rapid monitoring of scald disorder development. Several methods for the prevention of this phenomenon have been already proposed, such as low-oxygen controlled atmosphere storage, forced ventilation and 1-methylcyclopropene (1-MCP) treatment (Lurie and Watkins, 2012). The efficacy of these strategies is, however, influenced by maturity stage and uniformity of fruit batch. Maturity stage at harvest is indeed one of the major factors influencing the fruit susceptibility to scald. In fact, mature fruits have reduced scald sensitivity compared to immature ones (Wilkinson and Fidler, 1973; Wang and Dilley, 1999; Whitaker et al., 1997). However, all these methodologies lead to higher input and management costs.

The aim of this research was to develop an alternative, rapid, reliable analytical technique based on PTR-ToF-MS (proton transfer reaction – time of flight – mass spectrometry) to assess the volatile compounds (VOCs) involved in the superficial scald disorder in different apple tissues during storage, and following 1-MCP treatment.

MATERIAL AND METHODS

Plant Material and Growing Conditions

The apples used in this study were collected from five-year-old ‘Granny Smith’ apple trees grafted on M.9 rootstocks. The orchard was realized following a planting scheme of 3.8×0.8 m, and located at Bagnacavallo (Ravenna) in northern Italy. Standard cultural practice and disease management strategies were applied.

Fruit Selection and Storage Condition

Apples were harvested at a maturity stage assessed according to the method reported by Ziosi et al. (2008) and Nyasordzi et al. (2013). This maturity stage is represented by the I_{AD} index generated by the DA-Meter, a portable non-destructive device based on visible/Near Infra Red (vis/NIR) spectroscopy (TR, Forlì, Italy). I_{AD} usually ranges from 2.2 to 0, indicating the less ripe (thus characterized by a greater amount of chlorophyll) and the fully ripe apple fruit, respectively. For this investigation only fruits belonging to the I_{AD} class ‘1.8 -2’ were selected.

Homogeneous fruits (in both ripening stage and shape) were sampled immediately after harvest. Two apple batches, of about 80 fruits each, were distinguished: the first was used as the control while the second was treated with 1 ppm of 1-MCP. Treatment was applied for 24 h as SmartFreshTM (0.14% active ingredient), according to the manufacturer’s instructions (AgroFresh, Rohm and Haas, Philadelphia, Pennsylvania, USA).

Both apple batches were stored under normal atmospheric condition at +0.5°C and 95% relative humidity. Samples from the two batches were then removed after one and two months of cold storage, respectively. For both storage periods, additional sampling and scald incidence evaluation were performed after 1, 4, and 8 d of shelf-life at room temperature (around 20°C), to promote the incidence of the superficial scald on the apple fruit surface.

At each evaluation day, apple skin, under-skin and pulp tissues were assessed separately for each sample. Samples were represented by 10 randomly picked apples per treatment, and immediately frozen in liquid nitrogen and stored at -80°C prior to the analysis.

Sample Preparation

Powdered frozen samples (2.5 g) were immediately inserted into a 20-ml glass vial equipped with PTFE/silicone septa (Agilent, Cernusco sul Naviglio, Italy) and mixed with 2.5 ml of deionized water, 1 g of sodium chloride, 12.5 mg of ascorbic acid, and 12.5 mg of citric acid (for more details see Aprea et al., 2011). Samples were preserved at 4°C until the time of the analysis.

PTR-ToF-MS Analysis

Measurements of VOCs in apple tissues were performed in three replicates with a commercial PTR-ToF-MS 8000 apparatus (Ionicon Analytik GmbH, Innsbruck, Austria; Soukoulis et al., 2013). The conditions in the drift tube were the following: 110°C drift tube temperature, 2.25 mbar drift pressure, 550 V drift voltage. This leads to an E/N ratio of about 140 Townsend (Td) (E corresponding to the electric field strength and N to the gas number density; 1 Td=10⁻¹⁷ Vcm²). The sampling time per channel of ToF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to m/z = 400. Every single spectrum is the sum of about 28.600 acquisitions lasting 35 µs each, resulting in a time resolution of 1 s. Sample measurements were performed in 60 cycles resulting in an analysis time of 60 s/sample.

Each measurement was conducted automatically after 20 min of sample incubation at 40°C by using an adapted GC autosampler (MPS Multipurpose Sampler, GERSTEL) and it lasted for around 2 min. During the measurements, 100 sccm of zero air was continuously injected into the vial, through a needle heated to 40°C, and the outflow going through a second heated needle was delivered via Teflon fittings to the PTR-ToF-MS.

α-farnesene and 6-methyl-5-hepten-2-one identification was carried out through comparison of the PTR-ToF-MS fragmentation masses with pure standards.

RESULTS AND DISCUSSION

‘Granny Smith’ apples harvested at the ripening stage 1.8-2 (I_{AD}) were highly susceptible to superficial scald after two months of cold storage, showing 35 to 95% of fruit with visible scald symptoms after 4 and 8 d of shelf life at room temperature, respectively. Treatment with 1-MCP effectively reduced the incidence of scald to 0%, even after two months of cold storage. These data are in agreement with the previous studies carried out on the same apple cultivar by Lu et al. (2013), showing also that early harvested apples are generally more susceptible to scald (Bordonaba et al., 2013).

The majority of VOCs detected in the apple tissues collected here, showed a different accumulation upon storage condition and ethylene effect, as suggested by Schaffer et al. (2007). This fact was experimentally validated in this work by the application of the ethylene action inhibitor 1-MCP. Only a limited and specific subset of VOCs were associated with the incidence of the superficial scald, as suggested by Lurie and Watkins (2012). The entire VOC variability assessed among the skin samples were analysed by the means of a Principal Component Analysis (Fig. 1). The distribution depicted on the PCA plot clearly highlights the differences between control and 1-MCP treated samples as well as between the samples collected at variable stages. These differences were mainly attributed to known VOCs that have a direct influence on fruit quality such as esters aldehydes, alcohols, and ketones (Soukoulis et al., 2013). In the control skin samples, the separation between T1 and T2 stages, characterized by the presence of scald symptoms (in the latter stage) was mainly due to high concentration of α-farnesene and 6-methyl-5-hepten-2-one (MHO).

The main fragment masses of α-farnesene (m/z 205.195 and 149.114) and MHO (m/z 127.089 and 109.076) detected by PTR-ToF-MS analysis were also confirmed by using pure standards, showing a R² value of 0.99 and 0.97, respectively.

The α-farnesene and MHO contents were assessed in apple fruit skin, underskin, and pulp tissues during storage (Fig. 2). It is interesting to note the distinct behaviour between the two compounds assessed by PTR-ToF-MS. The concentration of α-farnesene (Fig. 2, left column) did not differ significantly throughout the time course. The accumulation of this compound, in fact, already started on the first day of shelf-life after 1 month of storage, resulting in a two-fold increase after only 3 days of shelf-life. From this point, the content of α-farnesene remained basically unchanged, showing only a slight increase in the stages assessed after two months of cold storage. The physiological dynamics of MHO was different (Fig. 2, right column). The concentration of this volatile ketone, originating from the oxidation of α-farnesene, showed a marked burst only in the

samples collected after two months of storage and this accumulation preceded the visual appearance of scald symptoms. During the postharvest shelf-life after one month of cold storage, the concentration of MHO remained similar to the concentration detected at harvest. After two months it increased, reaching its maximum 8 days under shelf conditions, showing an eight-fold increase in comparison with harvest levels. It is worth noting that the accumulation of MHO precedes the appearance of superficial scald symptoms, since MHO significantly changed in T2_1 samples, while scald impacted 35% of the samples in T2_4 stage. The different accumulation trends observed between these two compounds is consistent with their physiological pathways. The low concentration of MHO in the first month can be in fact attributed to the oxidation of α -farnesene. This oxidation process might be in fact the reason for the shift observed between the two compounds as depicted in Figure 2.

In light of these results, α -farnesene cannot be considered a reliable biological marker for superficial scald detection since its concentration rapidly increases with postharvest ripening, rather than with the occurrence of the scald symptoms. On the contrary, the accumulation trend of MHO was more specifically coincidental with the incidence of scald (as already evidenced by Mir et al., 1999 and Wang and Dilley, 2000), suggesting this as a more reliable compound for an early detection of the scald development. To further validate the efficacy of PTR-ToF-MS in predicting scald development in a real and competitive postharvest management, both α -farnesene and MHO were also successfully detected in the headspace of whole ‘Granny Smith’ fruits after two months of cold storage (Fig. 3).

In conclusion, we confirm the efficiency of 1-MCP treatment in the control of superficial scald development in ‘Granny Smith’ apple fruits by reducing the synthesis of ripening-related VOCs in the skin, especially α -farnesene and, consequently, its autooxidation product, MHO. Results of this investigation also pointed out i) the possibility to monitor VOCs involved in the superficial scald disorder by PTR-ToF-MS without the necessity to pre-extract samples with hexane or even in a non-destructive approach and ii) the opportunity to use MHO as a VOC marker to monitor oxidative stress processes of apples during storage and, in particular, to identify scald before the appearance of visible symptoms.

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Figures

Fig. 1. PCA distribution of VOCs assessed by PTR-ToF-MS analysis in control (●) and 1-MCP treated (●) 'Granny Smith' apples. Measurements were performed on skin tissue of fruit stored for 1 (T1) and 2 (T2) months of cold storage at +0.5°C, and then maintained for 1, 4 and 8 days of shelf-life at room temperature (~20°C). Each point is the average of 3 single measurements.

Fig. 2. Content of (a) α -farnesene (m/z 205.195) and (b) 6-methyl-5-hepten-2-one (m/z 127.089) in control (■) and 1-MCP treated (■) fruit of 'Granny Smith' apples during storage and shelf-life. Measurements were assessed by PTR-ToF-MS on "skin", "under skin", and "pulp" tissues of fruit left at room at temperature for 1, 4, and 8 days after 1 and 2 months (T1 and T2) of commercial storage at +0.5°C, respectively. Percentage of scald incidence (number of fruits with scald symptoms) and standard deviation (3 replicates) are shown.

Fig. 3. Quantification of α -farnesene (m/z 205.195) and 6-methyl-5-hepten-2-one (m/z 127.089) content in 'Granny Smith' apple fruit (control and 1-MCP treated) assessed by PTR-ToF-MS after 2 month of cold storage (+0.5°C). Measurements were done on headspace of intact fruit incubated for 30 min in a 5-L glass jar. Each data point is the average of 5 fruits. Bars indicate standard deviation.