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Citation for final published version:

Dhorajiwala, R., Roberts, C., Dimitrova, S., Baldwin, A., Davoli, D., Ludlow, R., Tu, S., Jones, S., Spadafora, N., Müller, C. and Rogers, H. 2021. Storage of halved strawberry fruits affects aroma, phytochemical content and gene expression. Presented at: 9th International Strawberry Symposium (ISS2021), Rimini, Italy, 01 - 05 May 2021. IX International Strawberry Symposium. Acta Horticulturae. , vol.1309 International Society for Horticultural Science (ISHS), 10.17660/ActaHortic.2021.1309.127

Publishers page: <http://dx.doi.org/10.17660/ActaHortic.2021.1309.12...>

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Storage of halved strawberry fruits affects aroma, phytochemical content and gene expression.

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Abstract

Strawberries are valued for their aroma and phytochemical content. However, they have a short shelf life and storage at low temperatures to prolong shelf life affects physiological and biochemical processes in the fruit. This impacts on their use in fresh cut ready-to-eat fruit salads. To assess changes in aroma and how these are related to phytochemical content and gene expression, *Fragaria x ananassa* cv. Elsanta strawberries were halved and stored at either 4 °C or 8 °C for a period of 12 days. Phytochemical content was relatively unaffected whereas volatile organic compound profiles were distinct at different time points of storage. Gene expression changed significantly with storage over a 5 day period: a total of 1135 gene targets changed in expression ($p < 0.05$; log2 fold change > 1.5) with most changes between days 0 and 5 of storage. These included genes related to stress responses, and secondary metabolism. Real time PCR was used to verify expression profiles of two genes related to VOC classes represented in the aroma, showing changes in pattern of expression during storage.

Keywords: *Fragaria x ananassa* Duch., transcriptomics, phytochemicals, volatile organic compounds

INTRODUCTION

The UK soft fruit market is worth approximately £473.3 million (2018) with strawberries representing over half of this value, worth £283 million (DEFRA, 2018). Cultivated strawberry, *Fragaria x ananassa* Duch. has been primarily bred for size and yield (Bertioli, 2019). This has resulted in the fruit being much larger than that of the wild species (*Fragaria vesca*), and with *Fragaria x ananassa* Duch. being octoploid rather than diploid (Edger *et al.*, 2019). The distinctive aroma of strawberry fruit is made up of a wide range of volatile organic compounds (VOCs) including esters alcohols, aldehydes, furanones, sulphur compounds and terpenes (Forney *et al.*, 2000; El Hadi *et al.*, 2013). Esters are thought play a key role in the aroma profile with many of them associated with high-quality fruit (Shamaila *et al.*, 1992; Pérez *et al.*, 1996). Some of the biosynthetic pathways generating VOCs in strawberry fruit are known. For example, FaOMT, an O-methyl transferase, catalyses the conversion of furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone) to mesifurane (2,5-dimethyl-4-methoxy-3(2H)-furanone) (Wein *et al.*, 2002). Furanones such as furaneol and mesifurane are important components of the strawberry aroma despite very low levels as both have low odour activity values (OAVs) with thresholds of 0.04 and 0.03 ppb detection respectively (Loehndorf *et al.*, 2000) and are essential for the characteristic strawberry aroma (Perez *et al.*, 1996). Cinnamyl alcohol dehydrogenase (CAD) catalyses the last stage in the phenylpropanoid pathway and may be involved in the production of aroma compounds in strawberry receptacle cells (Mitchell and Jelenkovic, 1995). As well as being valued for their taste, strawberries also contain high levels of phytochemicals with potential health benefits including phenolics, vitamin C and folate (Tulipani *et al.*, 2008; Giampieri *et al.*, 2012). The key phenolics in strawberry fruit include flavonoids, hydrolysable tannins and phenolic acids. Amongst these are quercetin, and its derivatives such as quercetin-3-O-rutinoside (rutin), and the flavonoid catechin.

Strawberry fruit has a short shelf life and is typically chilled during the supply chain from harvest to consumer (Mirzaee and Bishop, 2010). Chilling is used to slow down ripening and senescence postharvest (Pott *et al.* 2020). However, cold storage is associated with changes in fruit flavour related volatile organic compounds (Ayala-Zavala *et al.*, 2004; Forney *et al.*, 2000; Fu *et al.*, 2017). Phytochemical content, including vitamin C levels of berries postharvest can also be affected by storage conditions (Kårlund *et al.* 2015). However, in strawberries phenolics seem to remain relatively constant in chilled storage of at least some cultivars (Pelayo *et al.*, 2003).

Gene expression changes during strawberry fruit ripening (Li *et al.*, 2015; Sánchez-Sevilla *et al.*, 2017) as fruit softens and changes from green to red (Moya-León *et al.* 2019). Post-harvest gene expression is also affected by storage conditions (Zhang *et al.*, 2019). Some of the genes relating to the biosynthesis of VOCs have also been studied. For example, variations in the strawberry FaOMT locus are thought to cause variation in mesifurane content (Zorrilla-Fontanesi *et al.* 2012).

MATERIALS AND METHODS

Plant material

Strawberry fruit (*Fragaria x ananassa* cv. Elsanta) were grown outdoors at Hendrewennol Fruit Garden, Bonvilston, Cowbridge near Cardiff and harvested. Strawberry fruit homogeneous in size, colour (at red ripe stage) and with no external damage were handpicked and processed within 3 hours. Fruit were washed with water, the calyx removed using a sharp knife, immersed in 1 L of 200 ppm sodium hypochlorite solution for 2 min, air dried under sterile conditions, and chopped lengthways into halves using a sharp knife. The two halves were separated: one was stored at 4 °C and the other at 8 °C. Fruits were sampled destructively for VOCs before storage and then those stored at 8 °C were sampled at four further time points (1, 5, 7 and 12 days; Fig. 1). Controls were empty containers stored in the same way. For RNAseq and metabolite analysis halved fruit was stored at 8 °C and material collected at day 0, 1 and 5. Samples were rapidly sliced and snap frozen in liquid nitrogen then stored at -80 °C until used. Three replicates of 12 halved fruit were used for each sampling point.

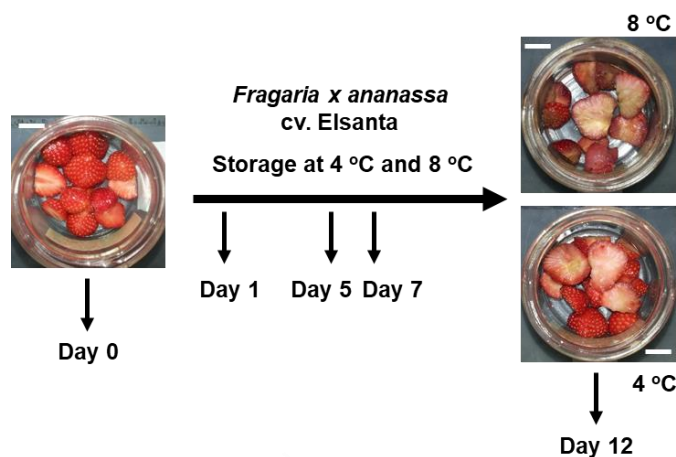


Figure 1. Experimental setup for strawberry shelf life experiment. Scale bar = 2cm

VOC collection and analysis

At each of the time points, VOCs were collected and processed essentially as described previously (Amaro *et al.*, 2018) with minor modifications including that fruit samples were equilibrated for 2 hours and 400 mL of headspace was collected. Desorption on a TD100 system (Markes International) was followed by GC MS analysis (GC7890A, Agilent Technologies, Inc., BenchTOFdx, Markes International). Data were processed with AMDIS

(NIST 2014) and ChemStation software (E.02.01.1177, Agilent Technologies, Inc.) as described previously (Amaro *et al.*, 2018).

RNA extraction and Transcriptomic analysis

Fruit tissue was ground to a fine powder under liquid nitrogen and 150 mg were used for extraction using a protocol adapted from Greco *et al.* (2014) based on CTAB extraction. Random primed cDNA libraries were prepared using a TruSeq RNA Sample Prep kit (Illumina) and Illumina sequencing was performed using paired-end mode on an Illumina Next Seq 5000 Platform. Reads were assessed using FASTQ, and Trimmomatic was used to remove all adaptor sequences, as well as leading and trailing low quality or N bases. Over 21 M reads were obtained for each sample. The *Fragaria vesca* Whole Genome v2.0.a1 was used as the reference genome (sourced from Rosecae.org 2017) and the *F. ananassa* sequences were mapped using Star.

Gene Ontology analysis

Plant Transcriptional Regulatory Map (PlantRegMap, Jin *et al.*, 2016) was used to analyse the significantly up-regulated or down-regulated genes from the RNA-seq data, both for the strawberry IDs already within the RNA-seq data set and Arabidopsis gene IDs which were assigned via a command line Blastx with the top hit being assigned. PlantRegMap analysis finds the significantly over-represented GO terms or parents of these terms from the input gene set. topGO and Fisher's exact tests are used by the analysis to obtain the GO terms that are significantly over-represented (p-value ≤ 0.01) in the input gene list.

Real Time PCR

Following removal of genomic DNA, cDNA was synthesised from 2 μ g of total RNA using MMLV reverse transcriptase (Promega). Primers for qRT-PCR specific for the O-methyltransferase (OMT) gene was from Carbone *et al.* (2006), and for the elongation factor-1 (EF1) gene from Amil-Ruiz *et al.* (2013), used as a control for transcript normalisation. The PCR consisted of 20 μ L of a mixture containing 10 μ L of SYBR Green (2x, PCR Biosystems), 0.4 μ L of each sequence-specific primer, 6 μ L of sample cDNA (10 μ M dilution). Each reaction was carried out in triplicate for each cDNA and gene. The PCR settings used were: 95 °C for 5 min, cycle x 40 of 95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec, then 60 °C for one min, 60 °C for 30 sec, 98 °C for 30 sec. Only Ct values with a range of 0.2 were used to normalise data and calculate relative expression.

Analysis of phenolics

Extraction and hydrolysis of phenolic compounds was adapted from (Tarola *et al.* 2013) using 0.25 g of frozen strawberry fruit. HPLC analyses were carried out on a ThermoScientific HPLC system (P4000 quaternary pump, AS300 autosampler and photodiode array detector (UV6000LP)) using a reversed phase C18 column with a 2 % formic acid in H₂O and 2 % formic acid in 90 % acetonitrile gradient (Tarola *et al.*, 2013). Calibration standards were used for quercetin, catechin, and rutin (from Sigma Aldrich, ≥ 95 %)

Statistical analysis

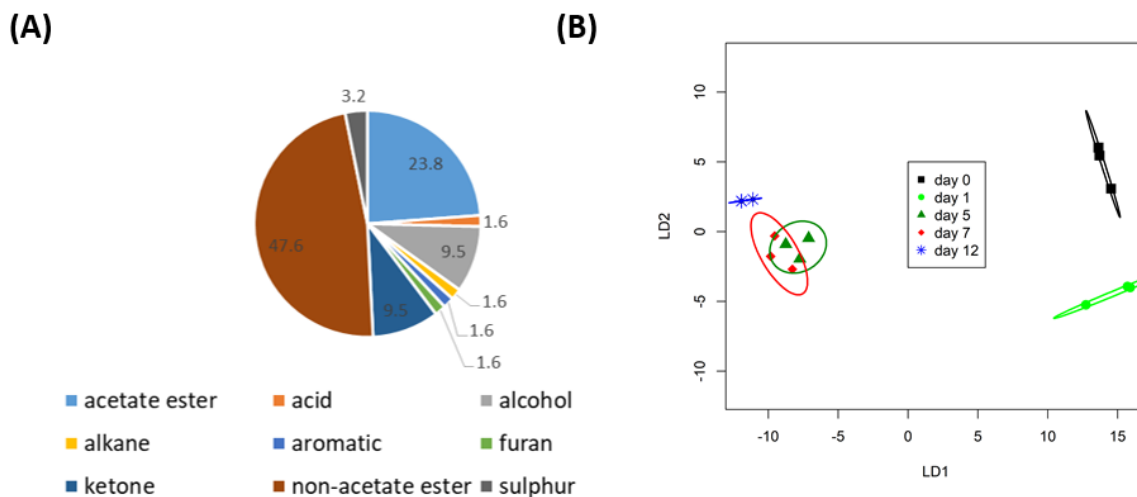
VOC profiles were analysed in R using Permutational Multivariate Analysis of Variance (PerMANOVA, adonis function in package vegan) and Canonical Analysis of Principal Ordinates (CAP, CAPdiscrim function in package BiodiversityR). Normality of datasets was tested using a Shapiro test, and equality of variance using a Fligner-Killeen test. Where datasets conformed to these tests (p > 0.05) a 2-way ANOVA was used followed by a Tukey's test. Where the data did not conform a Kurskal Wallis followed by a Dunn's test was used instead.

RESULTS

VOCs

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A total of 63 VOCs were detected in strawberries stored at 8 °C for up to 12 days (Fig. 2A). The greatest number were esters (acetate and non-acetate) representing over 70% of the total number, ketones and alcohols were the next most abundant VOC family with other VOC families representing a much smaller number of compounds in the profile. The whole VOC profiles were discriminated by day based on PerMANOVA ($p < 0.01$). A linear discrimination plot using the whole profile shows that there is clear discrimination between fresh cut fruit and fruit stored for 1 day (Fig. 2B). VOC profiles for fruit stored for 5 and 7 days were not



discriminated but VOCs on day 12 of storage were again discriminated from all other time points.

Figure 2- (A) % of VOC families represented (B) Canonical Analysis of Principal coordinates of the VOC profile of halved strawberries stored at 8 °C for up to 12 days. Each ellipse represents the 95 % confidence interval (SD). The plot uses linear discriminants LD1 and LD2 with a percentage of correct classification of 66.7 % ($n=3$)

Phytochemicals

Content of catechin, rutin and quercetin did not change significantly ($p < 0.05$) across the first five days of storage of halved strawberries at 8 °C, although there was a slight mean increase in catechin and rutin. At 5 days relative content was more variable across replicate fruit (Fig. 3).

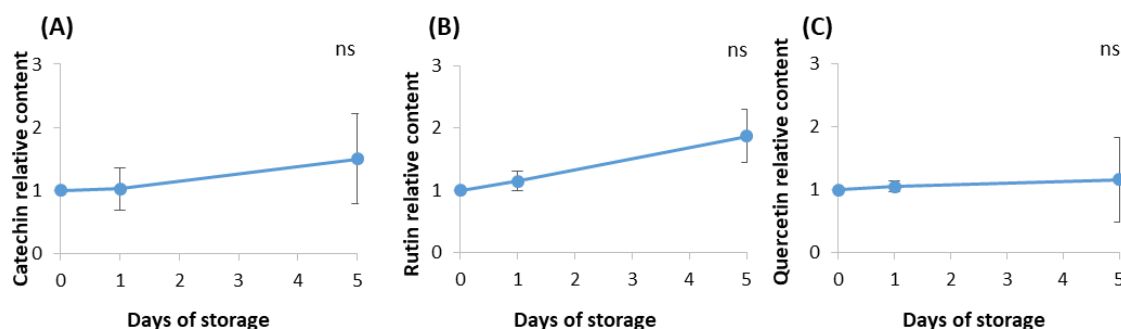


Figure 3 - Change in (A) catechin (B) rutin and (C) quercetin content in strawberry fruit that were halved and the stored at 8 °C for 5 days as assessed by HPLC (mean \pm SE; $n=3$ biological replicates).

Transcriptome

A total of 1135 genes were identified as differentially expressed ($p < 0.05$; \log_2 fold change >1.5) amongst the strawberry transcripts expressed on day of cutting, and after 1 and 5 days of storage at 8 °C. Most changes in gene expression were found in the comparison between day 5 and day 0 of storage (762 genes), lower numbers were changed between day 5 and day 1 (249 genes) and day 1 vs. day 0 (124 genes) (Fig. 4). There was most overlap in gene change between the day 5 vs day 0 and the day 5 vs day 1 than the other two comparisons.

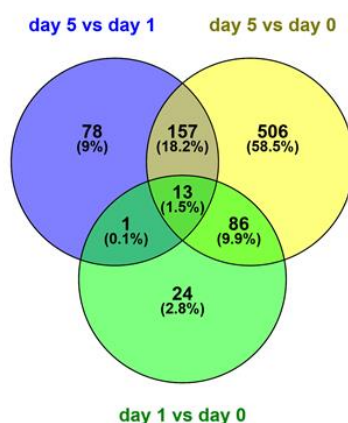


Figure 4- Changes in gene expression in pairwise comparisons amongst the three storage time points of strawberry fruit at 8 °C for 1 and 5 days (produced using Venny 2.0, <http://bioinfogp.cnb.csic.es/tools/venny/>).

Following functional annotation, 34-42 % of differentially expressed genes related to response to stress, and 14-22 % to secondary metabolism within GO terms relating to cellular processes. The comparison day 1 vs day 0 showed a higher % for both gene classes than day 5 vs. day 0 and day 5 vs. day 1 (Table 1).

Table 1 – Enriched differentially expressed genes (DEGs) as percentage total across three datasets from *Fragaria x ananassa* RNA-seq data relating to response to stress and secondary metabolism.

Day comparison and regulation	Number of genes		
	Response to stress	secondary metabolism	TOTAL
d1 vs d0	42	22	124
d5 vs d0	40	16	763
d5 vs d1	34	14	249

Real Time PCR

The effects of storage time and temperature on two genes related to the biosynthesis of VOCs, *FaOMT* and *FaCAD* was assessed (Fig. 5). *FaOMT* expression remained stable at both temperatures for the first day of storage but at day 5 there was significantly lower expression ($p < 0.05$) when the fruit were stored at 4 °C compared to 8 °C. Expression was also higher in fruit stored at 8 °C after 7 days compared to 1 day, while at 4 °C the rise in expression was only significant between day 5 and day 12. The pattern of *FaCAD* expression was different: expression at 4 °C peaked after 5 days. At 8 °C expression appeared to peak earlier at day 1 although there was variability across replicates, and the differences were not significant.

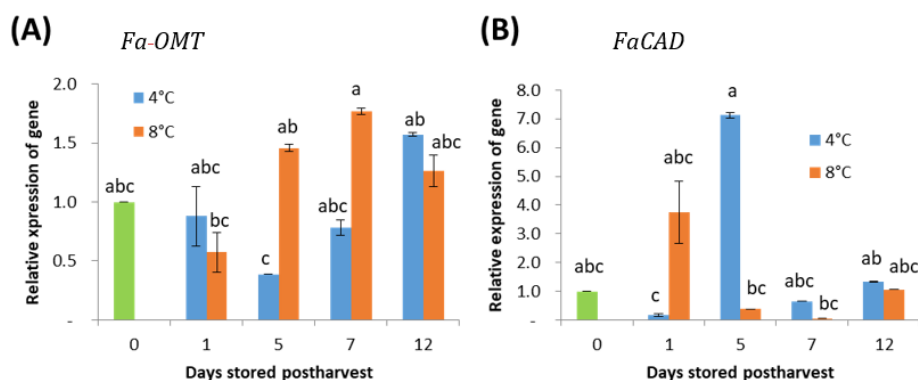


Figure 5- Comparison of change in relative expression of the strawberry (A) FaOMT and (B) FaCAD in fresh-cut strawberry over 12 days storage postharvest at 4 and 8 °C (n=3; + S.E.). Different letters indicate significant differences (p < 0.05).

DISCUSSION

The number of VOCs detected in this study is in line with other studies on individual varieties e.g. Van de Poel *et al.* (2014) who found 83 distinct VOCs in cv. Portola, although over 900 VOCs have been detected across strawberries in a wide range of different studies based on different detection methodologies (Ulrich *et al.*, 2018). The VOC families and relatively high number of esters found here is also in agreement with previous studies (Forney *et al.*, 2000; El Hadi *et al.* 2013; Ulrich *et al.*, 2018). Changes in VOCs during storage have also been previously noted (e.g. Ayala-Zavala *et al.*, 2004). Here we show that by using the whole volatilome we are able to clearly separate early stages of storage from later stages. By day 12 the strawberries had deteriorated well beyond their shelf life limit (Ayala-Zavala *et al.*, 2004) and VOCs were distinct from the mid-storage time points. The overall profile might therefore be of use in assessing strawberry quality and predicting shelf-life.

In contrast to VOCs, no significant changes were noted in the phytochemicals assayed here over the first 5 days of storage. This contrasts with some reports on total phenolics (e.g. Ayala Zavala *et al.*, 2004) which rose with storage at 5 °C, over a 12 day storage period, but is in line with other studies showing little change in phenolics during strawberry fruit shelf life (Pelayo *et al.*, 2003; Shin *et al.*, 2007). This may relate to cultivar differences or the specific storage temperature used.

The larger number of genes whose transcription changed in day 5 vs. day 1 compared to the genes that changed in expression between day 0 and day 1 probably reflects the longer storage period. However, the finding that a distinct set of genes is specific to each of these time intervals suggests that different processes are occurring early and later in shelf-life. During chilled storage, the fruit is subjected both to abiotic and biotic stress, as well as changing in ripeness, all of which affect their metabolism (Pott *et al.*, 2020). It is therefore perhaps not surprising that a high proportion of the changed genes during post-harvest storage relate to stress responses. Interestingly a higher proportion of stress related genes change early in post-harvest compared to later. The high proportion of stress related genes whose expression changes in the first day of storage may relate to a wound response, known to change metabolites in strawberry fruit (Hamilton-Kemp *et al.*, 2003) as well as an early response to chilling.

Numerous genes relating to secondary metabolites also change during chilled storage. This fits with studies showing changes in secondary metabolites during storage (Pott *et al.*, 2020) and also changes in the expression of genes relating to secondary metabolites. For example expression of the strawberry gene encoding cinnamate: CoA ligase (*FaCNL*) fell rapidly in the first 2 days of storage (Fu *et al.*, 2017), although other genes relating to aroma production such as *FaOMT* only changed in expression after 5 days of storage. In this study *FaOMT* expression also rose after about 5 days of storage at the higher storage temperature. The later expression when fruit were stored at the lower temperature could be due to the reduction in

metabolism associated with the increased cold (Pott *et al.*, 2020). A similar pattern was seen in the expression of FaCAD that rose earlier at the higher compared to the lower storage temperature. *FaCAD* is known to have maximum expression when strawberry fruit is mature (Blanco-Portales *et al.* 2002), so the increase here during storage may relate to the ripening which is slowed down at the lower storage temperature.

Conclusion

Overall we have shown that VOC profiles can be used to assess strawberry fruit shelf-life, and that there are significant changes in gene expression through shelf-life. Future work will focus on understanding the mechanisms underlying the changes in gene expression and VOCs during post-harvest storage, enabling better control of fruit quality.

ACKNOWLEDGEMENTS

RD, AB and RL were funded by the BBSRC, DD was funded by the ERASMUS+ programme, CR, SD ST and SJ were funded by Cardiff University, NDS was funded by FP7/KBBE2011.2.4-401/289719 – QUAFFETY – “Comprehensive approach to enhance quality and safety of ready to eat fresh products”.

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