Scientia Horticulturae 225 (2017) 150–155

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Changes in fatty acid and tocopherol content during almond (*Prunus dulcis*, cv. Nonpareil) kernel development

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ARTICLE INFO

Keywords:
Almond
Fatty acids
Fruit development
Lipids
Prunus
Tocopherols

ABSTRACT

Lipids are the major nutritional component of almonds and almond lipids comprise a range of fatty acids from C14 up to C20, including saturated, monounsaturated and polyunsaturated fatty acids, and oil soluble compounds such as plant sterols and tocopherols. This study investigated the change in fatty acid and tocopherol levels during almond kernel maturation, in the variety Nonpareil, grown in the Adelaide Plains of South Australia. The investigation was carried out between November 2012 and February 2013. The accumulation of lipids was determined over six timepoints, commencing at 74 days post-anthesis, and then at 20 day intervals. Almond lipid accumulation occurred rapidly between 95 and 115 days post-anthesis, i.e. at a rate of up to 1.83 g/day per 100 g fresh weight but then slowed. Tocopherols accumulated steadily and were positively correlated with lipid development; with α-tocopherol forming at the highest rate, being 0.58 mg/day in 100 g lipid, between the first two timepoints. The key timing for accumulation of the major fatty acid, oleic acid, was between 95 and 115 days post-anthesis, after which accumulation remained constant, at 0.57% of total lipids per day. In contrast, linoleic acid accumulated during the first two timepoints then declined to 23% of final lipid content. This study aimed to determine the timing of almond lipophilic antioxidant production, to inform almond orchard management practices, such as irrigation and fertilisation, which may impact kernel composition, and therefore, quality.

1. Introduction

Lipids represent the major nutritional component of almond kernels and account for more than 50% of total kernel dry weight (Kodad et al., 2011a; Zhu et al., 2015a,b). Isotope labelling experiments have previously been employed to study changes in the composition of lipids and fatty acids in almonds during development (Cherif et al., 2004; Munshi and Sukhija 1984; Soler et al., 1988). These studies, using [1-^{14}C] acetate incorporation, or organic solvents to extract fatty acid and triacylglycerol, monitored almond fatty acid biosynthesis. However, these studies were based on cultivars and development stages for almonds grown under northern hemisphere climatic conditions. So far, studies concerning the accumulation of fatty acids during almond kernel maturation have not been undertaken in the southern hemisphere where almond fruits are exposed to more solar radiation during maturation (Zhu, 2014 PhD thesis), in particular, solar UV radiation in the southern hemisphere is stronger than the northern hemisphere (Gies et al., 2004). Australia has a long history of almond production, and Australian production has increased dramatically over the last decade from 16,000 t in 2006 to over 81,000 t in 2016 (ABA, 2016). Australian almond producing regions experience unique environmental conditions, for example, limited rainfall (and frequent droughts), intense ultraviolet radiation (UVR), and predominantly red loamy and sandy soils, i.e. conditions which influence almond kernel development (Mousavi and Alimohamadi, 2006). Kodad et al. (2010) also pointed out the climatic conditions prevalent during the growing season, along with genotype and environment together influence almond oil content and fatty acid composition. It is therefore worth studying the changes in fatty acid profiles of Australian grown almonds during kernel development.

To date, the accumulation of tocopherols during almond lipid maturation has not been reported in the literature. Among the various tree nuts, almonds have the highest vitamin E (tocopherol) content (Kodad et al., 2011b; Zhu et al., 2015a,b). Tocopherol concentration is therefore a key nutritional measure of almond kernel quality. Almond lipids predominantly comprise the monounsaturated fatty acid, oleic acid, and the polyunsaturated fatty acid, linoleic acid (Kodad et al., 2011a; Zhu et al., 2015a,b) together with tocopherols collectively, these constituents have been shown to play an important lipophilic antioxidant role in human metabolism (Damasceno et al., 2011; Hollis and Mattes,

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http://dx.doi.org/10.1016/j.scienta.2017.07.008
Received 11 April 2017; Received in revised form 3 July 2017; Accepted 4 July 2017
Available online 11 July 2017

2007; Rajaram et al., 2010; Wien et al., 2010). The concentrations of tocopherols in fully ripened almond kernels has been well documented (Kodad et al., 2011b; Kornsteiner et al., 2006; Lopez-Ortiz et al., 2008; Madawala et al., 2012; Matthäus and Ozcan, 2009), but the accumulation of tocopherols during kernel development has not been extensively studied. In this study, changes in four tocopherol homologues over six stages of almond kernel development were followed, to determine the key timing of tocopherol formation.

Given the nutritional importance of lipids, unsaturated fatty acids and tocopherols, insight into their accumulation during almond kernel maturation might be used to inform the timing of almond orchard management practices, such as irrigation and fertilisation, in order to enhance kernel quality. Nanos et al. (2002) found irrigation enhanced oleic acid content in almond lipids compared with no irrigation. However, our previous study (Zhu et al., 2015a,b) observed that moderate deficient irrigation increased oleic acid in comparison to the control. Therefore, the present study aimed to investigate the key time points for almond lipids during drupe maturation, providing useful data for future studies. This study was performed on Nonpareil almonds, a cultivar grown extensively throughout Australia, as the basis for decision-making in the orchard.

2. Material and methods

2.1. Plant materials

Almonds were harvested from 26-year-old trees (*Prunus dulcis,* cv. Nonpareil) grown in an orchard in the North Adelaide Plains (34°92′S, 138°60′E, elevation 48 m above sea level), during the 2012–2013 growing season; with the orchard managed according to typical commercial practices. The soil comprised red-brown earth, with a high clay content. Climate data (Table 1) was sourced from the Australian Bureau of Meteorology (www.bom.gov.au). Almonds were sampled at six different timepoints starting at 74 days post-anthesis (t = 1) and then at approximately 20 day intervals thereafter (i.e. t = 2, 3, 4 and 5), until commercial maturity (t = 6). Two almonds were randomly selected from each of 40 trees at each timepoint. Kernels were opened and photographed with a Canon EOS500 digital camera. Kernels collected at t = 1, 2, 3, 4 and 5 were ground to a slurry and analysed in fresh form only; while fully ripened kernels (sampled at t = 6, i.e. 167 days post-anthesis, at commercial maturity when the mesocarp of almond drupes were dry and split, which denotes the almond fruit is fully mature), were analysed in both fresh and dried forms. Kernels were dried by heating at 50 °C for 48 h, to achieve a final moisture content of approximately 2%, measured according to the gravimetric technique (Zhu et al., 2015a,b). Dried kernels were ground to a fine powder with a coffee grinder, then sieved through a 1000 μm mesh, prior to compositional analysis.

2.2. Chemical reagents

Analytical grade hexane, ethanol, methanol, chloroform, *n*-heptane, sodium chloride, butylated hydroxyanisole (BHA), sulphuric acid, ascorbic acid and potassium hydroxide were purchased from Merck (French Forest, Australia), Scharlau (Gillman, Australia) and Sigma Aldrich (Castle Hill, Australia). A C17 free fatty acid (> 99% purity) was sourced from Nucheck Prep Inc. (Elysian, MN, USA) and used as an internal standard for determining the fatty acid profile of almond lipids. For identification and quantification of tocopherols, external standard curves were developed using an α, β, γ, δ-tocopherol standards set and an α-tocotrienol standard, sourced from Calbiochem (San Diego, CA, USA) and Cayman Chemicals (Ann Arbor, MI, USA), respectively.

Table 1
Climatic conditions in the Adelaide Plains during the 2012/13 growing season.

Year	Month	T_{max}[a] (°C)	T_{min}[a] (°C)	Rainfall[a] (mm)	Solar Radiation[a] (MJ/m^2)
2012	September	19.1 (18.3)	9.0 (8.9)	21.6 (54.4)	16.7 (15.5)
	October	21.9 (21.0)	9.6 (10.6)	15.6 (44.9)	23.4 (20.6)
	November	26.6 (24.0)	14.5 (12.8)	16.4 (30.5)	28.9 (24.7)
	December	27.0 (25.7)	15.5 (14.5)	13.6 (27.4)	30.3 (26.7)
2013	January	28.5 (28.1)	15.7 (16.0)	9.0 (21.2)	27.6 (27.7)
	February	28.7 (28.1)	17.3 (16.2)	12.4 (20.7)	23.7 (24.4)

Data from the Bureau of Meteorology website (www.bom.org.au).
[a] Seasonal data (and long term average data).

2.3. Fatty acid determination

Lipid extraction and fatty acid determinations were performed (in triplicate) using chloroform-methanol extraction and methanol-sulphuric acid FAME formation (fatty acid methylation), based on methodology previously described by Makrides et al. (1996) with some modification (Zhu et al., 2015a,b). Briefly, almond powder (0.05 g) was mixed with 0.9% aqueous sodium chloride (2 mL), methanol (3 mL, containing 0.005% BHA), C17 free fatty acid (400 μL, 0.16% in methanol) as an internal standard and chloroform (6 mL), and allowed to stand for 1 h. After extraction, samples were centrifuged (3000 × *g* for 10 min) and the organic phase separated and concentrated using a nitrogen evaporator (N-EVAP 112, Organomation Associates Inc., Berlin, MA. USA) at 45 °C. After evaporation, the vial containing the extract was weighed, and the difference between the vial with extract and the initial empty vial is the amount of the sample lipid. After drying, methylation was achieved by adding chloroform:methanol (9:1 v/v, 1 mL, containing 0.005% BHA) and methanol (5 mL, containing 1% sulphuric acid), and heating to 70 °C for 3 h. After samples had cooled, *n*-heptane (2 mL) and water (0.75 mL) were added and samples were mixed thoroughly. The organic layer was transferred to a GC vial for analysis. Fatty acid composition was determined using an HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA. USA) equipped with a flame ionisation detector (FID) and HP 7683 autosampler. Separation was performed on an SGE BPX 70 capillary column (50 m, 0.32 mm ID, 0.25 μm; SGE Analytical Science Pty. Ltd., Ringwood, Vic., Australia). Helium was used as the carrier gas and the split-ratio was 20:1. The injector temperature was 250 °C and the detector temperature was 300 °C. The initial oven temperature was 140 °C, increasing to 220 °C at 5 °C/min, and then held at this temperature for 3 min. FAMEs were identified and quantified based on the retention time and peak area of the C17 free fatty acid internal standard.

2.4. Tocol determination

Tocol extraction was based on the alkaline saponification and hexane extraction method used previously for analysis of cereals and nuts (Xu 2002) and described previously (Lampi 2011, Lampi et al., 2008). Briefly, almond powder (0.25 g) was mixed with ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% aqueous potassium hydroxide solution (0.25 mL). After being vortexed for 30 s, the samples were incubated in a water bath at 70 °C for 30 min, with (vortex) mixing at 10 min intervals. Samples were then placed in ice water for 5 min, before water (1.5 mL) and hexane (2.5 mL) were added, the resulting mixture vortexed for 30 s. Samples were then centrifuged (1000 × *g* for 10 min). The hexane layer was transferred to vials and the residue extracted again, before the combined hexane extracts were concentrated using a nitrogen evaporator (N-EVAP 112) at (45 °C). The resulting residue was re-dissolved in hexane (1 mL) prior to HPLC analysis, using previously published protocols (Lampi 2011; Lampi et al., 2008); i.e. the isocratic mobile phase was hexane (with 2% 1,4-dioxane), with a flow rate of 1.0 mL/min, an injection volume of 20 μL and column temperature of 25 °C. HPLC analysis was performed using an Agilent 1200 HPLC (Agilent Technologies, Waldbronn, Germany) coupled with diode array and fluorescence detectors (DAD and FLD, respectively).