Almond (*Prunus dulcis* (Mill.) D.A. Webb)  
Fatty Acids and Tocopherols  
under Different Conditions  

Ying Zhu  
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ABSTRACT

The thesis, comprising four articles (submitted and ready to be submitted), introduction with literature review and conclusion, is presented for a Degree of Doctor of Philosophy. The four journal articles represent four parts of a study on almond (*Prunus dulcis* (Mill) D.A. Webb) fatty acid and tocopherol composition and the influence of different growing conditions, including regionality and variety, solar ultra violet radiation (UVR) and drought.

Fatty acids and tocopherols are key nutrients which give almonds various therapeutic functions in many health aspects, thereby, almond fatty acids and tocopherols are very important for industry and researchers alike. Based on this significance, the current project aimed to investigate: regional and variety differences particularly in Australian growing regions and across Australian almond breeding selections; solar UVR effects; and the influence of deficit irrigation and lipid maturation on tocopherol accumulation during almond fruit ripening.

The regional and varietal study showed that genotype plays a greater role in differentiating almond unsaturated fatty acids (USFA) and tocopherols than environment. The study also found that irrigated regions predominant in Australia and California produced higher linoleic acid concentration in almonds than rainfall dependant regions like Spain and other Mediterranean countries. The results also demonstrated that selections No. 13 and No. 23 from the Australian almond breeding program had high Vitamin E content and oleic/linoleic acid ratio (O/L ratio) comparable with the variety Guara and Somerton, but has a more pleasing appearance for marketing promotion.

The investigation into the influence of solar UVR showed that a medium dose of increased solar UVR from reflective white weed mat below trees enhanced almond tocopherol concentration, i.e. 14% solar UVR increase enhanced almond tocopherol concentration by
30%. The increased solar UVR did not influence almond lipid content but slightly decreased linoleic acid concentration (i.e. by 2%) and increased oleic acid concentration (by 1.5%).

An examination into the effect of deficit irrigation on almond composition demonstrated that moderate water deficiency i.e. 85% of Evapotranspiration (ET$_o$) irrigation did not impact on almond lipid and tocopherol concentration, and fatty acid composition was unchanged. In order to gain such circumstances, the deficit irrigation needs to be sustained during the irrigation period, rather than up and down in a periodical way.

By studying almond composition during fruit development, almond lipid maturation and tocopherol synthesis was found to predominantly occur in the early stages of fruit ripening; i.e. 95 to 115 days and 74 to 95 day after anthesis, 1.83 g /day per 100g almond and 0.58 mg /day in 100 g lipids, respectively. On average, each kernel weighs 1.0 g, then during the time of 95 to 115 days and 74 to 95 day after anthesis, daily lipid accumulation in each kernel was 1.83 mg and daily tocopherol synthesis in each kernel was 2.2 ng.

In summary, this project concerns academic research and industry interests alike. The results are expected to be useful for almond orchard management in aspects of irrigation, fertilization and spraying, in order to control and improve almond kernel quality, as well as to provide new information to the broader horticultural research area.
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SYMPOSIA


2. 3 MT competition Jul. 2013, the winner of School of Agriculture, Food and Wine and a finalist of Faculty of Science heat in Aug. 2013.

3. 27-31 May 2013, VI Symposium International on Almonds and Pistachios in Spain Murcia, Conference paper presentation ‘Effect of deficit irrigation on almond kernel constituents’.


5. 18-20 Sept. 2012, Annual Waite Symposium, *oral presentation* ‘Regional and varietal study on almond tocopherols and fatty acids’.


7. 1-3 Feb. 2012, Australian Food Science Summer School by Australian Institute of Food Science and Technology Incorporated, in Melbourne, *oral presentation* ‘Nutrient-intense source, prospect of almonds on food processing’.


11. 16-17 May. 2010, AIFST Dietary Fibre and Health Conference in Adelaide, participation and discussion.

LIST OF ABBREVIATIONS

BHA butylated hydroxyanisole
DAD diode array detector (coupled with HPLC)
ET<sub>o</sub> evapotranspiration
FA fatty acid
FFA free fatty acid
FID flame ionisation detector (coupled with GC)
FLD fluorescence detector (coupled with HPLC)
GC gas chromatography
GLC gas liquid chromatography
HDL high-density lipoprotein
HPLC high performance liquid chromatography
LDL low-density lipoprotein
MED minimal erythemal dose (200Jm<sup>-2</sup> for skin type I)
Me-OH methanol
MUFA monounsaturated fatty acid
O/L ratio of oleic acid to linoleic acid
PUFA polyunsaturated fatty acid
SFA saturated fatty acids
UVR ultraviolet radiation
USFA unsaturated fatty acids
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CHAPTER ONE
General Introduction and Literature Review

The almond, *Prunus dulcis* (Mill) D.A. Webb, is one of the popular tree nuts in western diets. Nowadays, it is highly appreciated by many different food culture communities worldwide, partly because of the nutritional content of the kernel. Originally, almond trees were grown in central and south-western Asia, in particular the Kopet Dagh mountain range between Iran and Turkmenistan, as well as in the Tien Shan Mountains between Kyrgyzstan and western China (Janick and Paull 2008). During early civilization, along with other crops such as grapes, olives and figs, almonds were brought through the major trade routes into the Mediterranean region in particular Italy, Spain and Morocco. The almond boom in the Mediterranean lasted quite a long time through the western culture progression and expansion till early colonists settled in America. In the late nineteenth century, almond trees were brought to California and cultivated and developed there under similar Mediterranean climatic conditions. Almond global dissemination had started the journey and achieved a successful performance at a global industrial scale until the end of the twentieth century (Rosengarten 1984, Kester and Grasselly 1987, Micke 1996, Janick and Paull 2008). Having enjoyed the global money–spinning marketing for half a century, the Californian almond industry faced a challenge from southern growing regions when the 21st century began. It is the Australian almond industry, small, but dynamic. In 2011, it surpassed Spain and became the second largest producer, making up 3.3% of the worldwide production (Australian Almond Statistics Report 2012); in 2012, it rose to 4.3% of global production (Australian Almond Statistics Report 2013).

The Australian almond industry has rapidly grown in the last two decades (Gilbert 2005, Wilkinson 2012). Along with the wine industry boosted by the Asian emergent economy, the
Australian almond industry has found its niche in the global market and further extended to the traditional European market. The climate along the Murray river corridor is similar that of Mediterranean and the most suitable to plant almond trees and establish orchards in the four growing regions of Australia include Sunraysia in Victoria, Adelaide and Riverland in South Australia, Riverina in New South Wales (Australian Almond Strategic R & D Plan 2011-2016). Currently, the Australian almond industry produces more than 70,000 tonnes of kernels annually: 60% for export with the major markets including India (39%), United Arab Emirates (13%), New Zealand (8%), China (3%) and Saudi Arabia (3%); most popular varieties in the market consisting of Nonpareil (51%), Carmel (32%), Price (12%), Peerless (1%) and Ne Plus (1%) (Australian Almond Strategic R & D Plan 2011-2016).

The factor behind this almond boom is the high nutrition value as perceived by consumers. Almonds are a nutrient-dense source with respect to lipids, protein, dietary fibre and minerals. Almonds have been measured regarding the following compounds (Yada et al. 2011):

- **Protein**, ranging from 16% to 22% of kernel weight between varieties, with the major type globulin (app.74%) and albumin (app.21%);
- **Amino acids**, mainly bound in almond protein-amandin, with low content of free amino acid (less than 200mg/100g);
- **Lipids**, accounting for more than 50% of kernel weight, with the majority of unsaturated fatty acids, oleic (app. 70%) and linoleic (app. 20%);
- **Vitamins**, lipid-soluble (lipophilic) E type excessively in rich, with the major homologue α-tocopherol, and other minor homologues γ, β-tocopherols, α-tocotrienol; water-soluble (hydrophilic) B type in small amount (thiamine B\textsubscript{1}, riboflavin B\textsubscript{2}, niacin B\textsubscript{3}, pantothenic acid B\textsubscript{5}, pyridoxine B\textsubscript{6}, biotin B\textsubscript{7}, folate B\textsubscript{9}).
- **Dietary fibres**, making up 10.8% - 13.5% of the total kernel, including pectin, cellulose and xyloglucans, while starch and sugar are very low in content;
- **Minerals**, a range of minerals such as calcium, iron, magnesium, phosphorous, potassium, zinc, manganese and selenium, especially high in potassium (705 mg/100 g) and phosphorus (484 mg/100 g).
Of these nutrients, lipids and Vitamin E feature in the health profile for almonds, relating to high lipophilic anti-oxidation properties. Lipids are the major nutrients in almond, in both quantity and quality, i.e. lipids account for more than 50% of the total kernel weight in which approximately 90% is unsaturated fatty acids (USFA). In general, almond lipids comprise oleic acid (C18:1) (approx. 65%) and linoleic acid (C18:2) (approx. 25%), palmitic acid C16:0 (approx. 6.5%), stearic acid C18:0 (approx. 2.5%), palmitoleic acid C16:1(approx. 0.7%), and trace amounts of myristic acid (C14:0) and arachidic acid (C20:0) (Figure 1). These fatty acids have been identified in a range of commercial varieties and selections from California, Spain, Italy, France, Greece, Ukraine, China, India, Morocco and Syria (Askin et al. 2007, Sathe et al. 2008, Kodad and Socías i Company 2008, Sanahuja et al. 2009, Moayedi et al. 2010, Piscopo et al. 2010, Kodad et al. 2010, Tian et al. 2011, Kodad et al. 2011b). The high proportion of the monounsaturated fatty acid (MUFA) (oleic acid C18:1) and polyunsaturated fatty acid (PUFA) (linoleic acid C18:2) are attributed to almond’s cardiovascular health benefits (Josse et al. 2007, Jenkins et al. 2008, Wien et al. 2010, Jaceldo-Siegl et al. 2011). Moreover, the abundance of Vitamin E in almond lipids actually plays an outstanding role portraying almond’s putative health profile. Vitamin E is the common name for the biological compounds tocopherols and tocotrienols. So far Vitamin E has been found to have 8 homologues, α, β, δ, γ-tocopherol and α, β, δ, γ-tocotrienol (Figure 2) (Kamal-Eldin and Appelqvist 1996). Of the eight homologues, α-tocopherol has been reported as having the highest biological activity, followed by γ-tocopherol (Rizzolo et al. 1994, Zacheo et al. 2000, Okogeri and Tasioula-Margari 2002). Previous research found that almond Vitamin E consists mainly of α-tocopherol (approx. 90%), γ-tocopherol (approx. 6%), with minor amounts of β, δ-tocopherol and α-tocotrienol (Maguire et al. 2004, Kornsteiner et al. 2006, Lopez-Ortiz et al. 2008, Mattheus and Ozcan 2009, Kodad et al. 2011a, Madawala et al. 2012).
myristic acid (C14:1)

palmitic acid (C16:0)

palmitoleic acid (C16:1)

stearic acid (C18:0)

oleic acid (C18:1)

linoleic acid (C18:2)

arachidic acid (C20:0)

Figure 1. Chemical structures of major fatty acids in almond lipids

α-tocopherol

β-tocopherol

γ-tocopherol

δ-tocopherol

α-tocotrienol

β-tocotrienol

γ-tocotrienol

δ-tocotrienol

Figure 2. Chemical structures of isomers of tocopherols and tocotrienols
Based on the high proportion of USFA and the abundance of tocopherols, almond health benefits have been scientifically proven by many clinical trials, pre-clinical trials and epidemiologic studies. Studies showed that regular almond consumption can significantly decrease LDL (low density lipoprotein) cholesterol, postprandial glycaemia, insulinaemia, improve body weight control and reduce the risk of obesity-related diseases, such as coronary disease and type II diabetes (Spiller et al. 1998, Fraser et al. 2002, Lovejoy et al. 2002, Jambazian et al. 2005, Hollis and Mattes 2007, Josse et al. 2007, Jenkins et al. 2008, Mandalari et al. 2008, Rajaram et al. 2010, Wien et al. 2010, Sabate et al. 2010, Damasceno et al. 2011, Jaceldo-Siegl et al. 2011). The actions of those compounds such as oleic acid, linoleic acid and tocopherols on the cardiovascular system metabolism have been explained as antagonizing LDL oxidation, inhibiting platelet aggregation and adhesion, reacting with oxidation agents such as free radicals in LDL and preserving coronary dilation (Kamal-Eldin and Appelqvist 1996, Jia et al. 2006). Two of these studies have demonstrated that almond lipids offer greater cardiovascular benefits than virgin olive oil: the daily consumption of 100 g of almonds was found to reduce LDL cholesterol by up to 12% in hypercholesterolemic patients, which exceeded the LDL reduction achieved with a daily intake of 48 g olive oil (Spiller et al.1998). Similarly, Damasceno and colleagues (2011) found LDL-cholesterol reductions of 13.4% were achieved with almond rich diets, compared to the 7.3% reductions achieved with an olive oil based diet. In addition to cardiovascular benefits, almond lipids can also enhance bioactivity in other tissues. For example, in pre-clinical trials almond oil provided therapeutic prevention against rat liver damage, demonstrated by increased levels of hepatic superoxide dismutase, catalase and glutathione (Jia et al. 2011). Almond consumption is also thought to ameliorate oxidative stress in the DNA of smokers (Jia et al. 2006).

Moreover, research found almond skin is rich in polyphenols, hydrophilic antioxidants including flavan-3-ols as such catechin, epicatechin; flavonol aglycones such as isorhamnetin,
kaempferol and quercetin; flavonol glycosides as such isohamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside and quercetin-3-O-glucoside; flavanone aglycones such as eriodictyol and naringenin; flavanone glycosides such as eriodictyol-7-O-glycoside and naringenin-7-O-glycoside; as well as other simple phenolic acids reported as 4-hydroxybenzoic acid, vanillic acid, protocatechuic acid, chlorogenic acid and trans-coumaric acid (Sang et al. 2002, Milbury et al. 2006, Hughey et al. 2008, Bolling et al. 2009, Yildirim et al. 2010).

Like most plant phenolics, research found almond skin polyphenols have antioxidant and anti-inflammatory bioactivity. For example, one of the phenolic compounds present in almond skins, protocatechuic acid, has a strong antioxidant effect, as much as 10-fold greater than that of α-tocopherol (Sang et al. 2002). Several studies have shown therapeutic functions of these polyphenols in almond skins targeting spinal cord injury (Mandalari et al. 2011a), hepatocytes in carbonyl stress (Dong et al. 2010), and lowering the risk of colorectal, colon and rectal diseases (Mandalari et al. 2008, Garrido et al. 2010, Llorach et al. 2010, Mandalari et al. 2010a, Mandalari et al. 2011b). Almond skin polyphenols occur as a range of flavonoids from aglycone forms to glycosides. The forms of aglycone have greater efficacy in antioxidant activity than that of the glycosides (Wijeratne et al. 2006).

In short, the increasing rate of almond consumption stems from consumer perception of almond health benefits which have been proven by many studies. Meanwhile, it has also opened more areas for further research including how to optimise and improve those health benefits. This PhD project focuses on lipids, fatty acids and tocopherols and aims to maximise the nutritional values by selecting the excellent source from different regions and varieties (optimising), and to enhance the amounts of those compounds by applying orchard management techniques.
The significance of the regional and varietal study on almond tocopherols and fatty acids in this project is to investigate these effects for the Australian almond industry, in order to provide academic data for the industry’s consideration. Many studies on regional and varietal differences of almond tocopherol and fatty acid have been done for northern-hemisphere regions such as Mediterranean and north-America, but not for southern-hemisphere Australian region. However, the climate, soil and seasonal differences contribute to the variation of almond tocopherol and fatty acid composition; therefore, it is worth carrying out the investigation for the Australian almond industry.

Moreover, water saving solutions are very important techniques for Australian agricultural industries, given Australia has a shortage of water resources. The project of ‘Optimising water use of Australian almond production through deficit irrigation strategies’, was initiated by DEPI Victoria in collaboration with Horticulture Australia Limited and the Almond Board of Australia, in an effort to establish a benchmark for water saving solutions for almond orchards under Australian inland climate. This part of the PhD project specifically examined the changes in almond lipids, fatty acids and tocopherols after deficit irrigation, in order to assess the kernel quality under water stress.

In addition, solar UVR is an environmental factor for agricultural crops. Research has found that moderate doses of UV radiation can stimulate the protective mechanisms in plants (Kakani et al. 2003), such as leaf thickness, epidermal wax and hairiness (Steinmuller and Tevini 1985; Bornman and Vogelman 1991; Nagel et al. 1998; Manetas 2003), and chemical compound changes like increasing flavonoids (Alexieva et al. 2001, Martz et al. 2007, Josuttis et al. 2010). The Australian continent experiences 12-15% higher solar UVR than similar
latitudes in northern hemisphere (Gies et al. 2004). Therefore, it is necessary to investigate how solar UVR influences almond antioxidants such as tocopherols.

Finally, given the significance of tocopherol and fatty acids in almond nutritional value, to explain the developing portfolio of those compounds during almond kernel maturation is very valuable research, because it could be useful for almond orchard management practices such as irrigation, fertilization and other kernel quality improvement purposes.
CHAPTER TWO
Influence of Region and Variety on Fatty Acid and Tocopherol Concentration of Almond

Introduction

Fatty acids and tocopherols are important nutrients that impart health benefits to almonds (Mandalari et al. 2008, Rajaram et al. 2010, Sabate et al. 2010, Wien et al. 2010, Tian et al. 2011) and the concentrations of these nutrients are often used as selection criteria by industry, for example in the development of new cultivars or determination of the suitability of growing regions (Wirthensohn & Sedgley 2002, Kodad et al. 2004, Socia i Company et al. 2010, Kodad et al. 2011b). Previous studies have shown that the content and composition of fatty acids and tocopherols varies according to almond variety and growing region (Sathe et al. 2008, Kodad et al. 2010).

Traditionally, almonds have been grown in Mediterranean regions, such as Spain, Italy and Morocco, where different provinces tend to favour particular almond varieties. In California, Nonpareil is the predominant variety and it has been widely planted for more than 100 years, albeit other varieties allow for diversification of American almond production. The influence of region and variety on the fatty acid and tocopherol concentration of almonds grown in the northern hemisphere regions has been well studied (Sathe et al. 2008, Kodad et al. 2010, Yada et al. 2013). In contrast, comparatively little is known about the fatty acid and tocopherol composition of almonds grown in different regions in Australia. The Australian almond industry currently grows Nonpareil, Carmel, Peerless, Price and Guara almond varieties, which were introduced from California and Spain; but several local varieties have been developed, such as Johnston, Chellaston and Somerton and are also grown (Almond Board of
Australia 2012a). Over the last decade, the Australian almond industry has increased its share of the global market, to the delight and fiscal benefit of domestic growers (Wilkinson 2012b). As a consequence, there is considerable interest from industry in the development of new cultivars; in particular, cultivars which yield almonds of superior nutritional content.

The following paper describes an investigation into the fatty acid and tocopherol concentration of several varieties of almonds grown in different almond-producing regions of Australia, as well as almonds grown in Spain and California. Understanding the influence of region and variety on almond composition will enable industry to make informed decisions with regards to almond breeding programs and the suitability of almond cultivars to particular growing regions.
Paper: Lipophilic Antioxidant Content of Almonds (Prunus dulcis): A Regional and Varietal Study

Ying Zhu, Kerry Wilkinson and Michelle Wirthensohn*

The University of Adelaide, School of Agriculture, Food and Wine, PMB 1 Glen Osmond,
SA 5064, Australia

Email for Correspondence: michelle.wirthensohn@adelaide.edu.au

(Submitted to the Journal of Food Composition and Analysis)
ABSTRACT

This study investigated fatty acid and tocopherol of 24 almond samples (*Prunus dulcis*) mainly sourced from several regions in Australia, as well as Spain and the USA, to determine the influence of variety and region on linoleic acid and Vitamin E content in particular. Considerable variation was observed not only in linoleic acid (15.7% – 29.9% of total lipids) and Vitamin E (8.2 mg – 21.5 mg/100 g) content, but also lipid (46.1 – 63.5 g/100 g), oleic acid (58.5% – 71.3% of total lipids), palmitic acid (5.9% – 7.5% of total lipids) and stearic acid (1.0% – 2.4% of total lipids) content. Tocol composition comprised α-tocopherol (8.0 – 20.9 mg/100 g), γ-tocopherol (0.08 – 0.59 mg/100 g), β-tocopherol (0.02 – 0.12 mg/100 g) and α-tocotrienol (0.01 – 0.30 mg/100 g). The influence of genotype and environment were evaluated and enabled identification of new selections, being No.13 and 23, as almonds suited to growing conditions in Australian regions.

KEYWORDS: almond, fatty acid, linoleic acid, lipophilic antioxidant, Vitamin E, tocopherol
INTRODUCTION

Almonds are a nutrient-rich source of lipids, protein, dietary fibre, minerals, vitamins and polyphenols (Esfahlan et al. 2010, Yada et al. 2011). The health benefits afforded by almonds have been largely attributed to lipids and vitamins, with numerous clinical and pre-clinical trials and epidemiologic studies showing that regular consumption of almonds can: significantly reduce low density lipoprotein (LDL), cholesterol, postprandial glycaemia and insulinaemia; improve body weight control; and reduce the risk of obesity-related diseases such as coronary heart disease (CHD) and type II diabetes (Spiller et al. 1998, Hyson et al. 2002, Hollis and Mattes 2007, Jenkins et al. 2008, Rajaram et al. 2010, Wien et al. 2010, Damasceno et al. 2011). For example, two of these studies have demonstrated that almond lipids offer greater cardiovascular benefits than virgin olive oil: the daily consumption of 100 g of almonds was found to reduce LDL cholesterol by up to 12% in hypercholesterolemic patients, which exceeded the LDL reduction achieved with a daily intake of 48 g of olive oil (Spiller et al. 1998); similarly, Damasceno and colleagues found LDL-cholesterol reductions of 13.4% were achieved with almond rich diets, compared to the 7.3% reductions achieved with an olive oil diet (Damasceno et al. 2011). In addition to cardiovascular benefits, almond lipids can also enhance bioactivity in other tissues. For example, in pre-clinical trials almond oil provided therapeutic prevention against rat liver damage, demonstrated by increased levels of hepatic superoxide dismutase, catalase and glutathione (Jia et al. 2011). Almond consumption is also thought to ameliorate oxidative stress in the DNA of smokers (Jia et al. 2006). All of these health benefits have been largely attributed to high proportions of the unsaturated fatty acids (USFA) oleic acid, linoleic acid and Vitamin E.

Linoleic acid, an omega-6 fatty acid, is an essential fatty acid for humans and is involved in children’s growth and the prevention of cardiovascular disease (Hollis and Mattes 2007, Jenskin et al. 2008, Wien et al. 2010). However, high levels of linoleic acid have been
considered as markers of almond spoilage, since linoleic acid’s double bonds are susceptible to oxidation, thus, high levels of oleic acid and low levels of linoleic acid have been associated with prolonged shelf-life of almonds and are often advocated (Kodad and Socías i Company 2008, Kodad et al. 2009, Zaplin et al. 2013).

However, another property in lipids, Vitamin E, can improve almond shelf-life (Socías i Company et al. 2010). Of the various tree nuts, almonds typically contain the most Vitamin E; with two handfuls of almonds providing the average daily recommended dose of Vitamin E, being 15 mg per day (Institute of Medicine 2000). Previous studies have shown almond Vitamin E largely comprises α-tocopherol and γ-tocopherol, with lesser amounts of β-tocopherol, δ-tocopherol and α-tocotrienol also present; the relative proportions of which are thought to be influenced by genotype and region of origin (Kodad et al. 2006, Kodad et al. 2009, Yildirim et al. 2010, Kodad et al. 2011a, Lopez-Ortiz et al. 2012). The tocopherol concentration of almond oil derived from either commercial or germplasm almonds typically ranges from 14.4 to 55.3 mg/100 g (Zacheo et al. 2000, Delgado-Zamarreno et al. 2004, Maguire et al. 2004, Kornsteiner et al. 2006, Kodad et al. 2009, Matthäus and Özcan 2009, Madawala et al. 2012).

The Australian almond industry dates back 100 years, during which time Australian growers have established local varieties such as Johnston, Chellaston and Somerton, in addition to the commercial varieties available from California and Spain which include Nonpareil, Carmel, Peerless, Price and Guara. Almond breeding programs have also developed new selections for industry. However, very little is known about the Vitamin E and fatty acid composition of almonds grown in different Australian regions. This study aimed to investigate the influence of environmental conditions (i.e. regionality) and genetics (i.e. variety) on almond fatty acids in particular linoleic acid and Vitamin E. Improved knowledge of the regional and/or varietal
differences in almond composition may enable industry to make more informed decisions with respect to varietal selection.

MATERIALS AND METHODS

2.1 Plant Material. Australian almond samples were collected from Willunga (35°17’S, 138°68’E, elevation 365 m), Adelaide (34°92’S, 138°60’E, elevation 48 m), Riverland (34°11’S, 141°02’E, elevation 59 m) and Sunraysia (34°24’S, 142°09’E, elevation 50 m) during the 2012 season. Spanish almonds were sourced from Zaragoza (41°39’N, 0°53’ W) and North American almonds were sourced from Merced (37°18’ N, 120°29’ W), California, also during 2012. The cultivars selected were: Nonpareil (from Sunraysia, Adelaide, Willunga, Riverland, Zaragoza and Merced), Johnston (from Adelaide and Willunga), Somerton (Willunga), Peerless (Riverland), Price (Riverland), Carmel (Riverland and Merced) and Guara (Riverland and Zaragoza). The selections from the Australian almond breeding program (Wirthensohn and Sedgley 2002) were: No.12, No.13, No.23, No.31, No.32, No.48 and No.53 (from Riverland). Samples (100 kernels randomly harvested per tree per cultivar or per tree per selection) were harvested when the mesocarps had naturally split, indicating ripening. For each cultivar, 25 trees from growers’ orchard were harvested and for each selection, five trees from the Riverland breeding field were harvested. Kernels were dried at 50°C for 48 hours and the moisture content (approximately 2%) confirmed by the gravimetric technique. Dried kernels were ground using a coffee grinder to a fine powder, sieved through a 1000 µM mesh and then stored under nitrogen prior to analysis.

2.2 Chemical Reagents.

C17 free fatty acid (C17 FFA from Nucheck Prep Inc.) was used as an internal standard to quantify the fatty acid percentage of almond lipids. For tocopherol identification and
quantification, an α, β, γ, δ-tocopherol standard set (Calbiochem, Germany) and an α-tocotrienol standard (Cayman Chemicals, USA) were used to develop external standard curves. Hexane, ethanol, methanol, chloroform, n-heptane, sodium chloride, butylated hydroxyanisole (BHA), sulphuric acid, ascorbic acid, potassium hydroxide were purchased from Merck, Scharlau and Sigma.

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 reported by Makrides and colleagues (Makrides et al. 1996), with some modification. Briefly, almond powder (0.05 g) was mixed with 0.9% aqueous sodium chloride (2 mL), methanol (3 mL containing 0.005% BHA), free fatty acid C17 (400 µL, 0.16% in methanol) as an internal standard and chloroform (6 mL), and then 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 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 hours. After the 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. Fatty acid composition was determined using an HP 6890 Gas Chromatograph (Hewlett Packard, Palo Alto, CA. USA) equipped with a flame ionization detector (FID), split/splitless injection, HP 7683 autosampler and HP Chemstation. A capillary GC column SGE BPX 70 (50 m, 0.32 mm ID, 0.25 µm) was used (SGE Analytical Science Pty. Ltd. RingWood, VIC. Australia). Helium was the carrier gas and the split-ratio was 20:1, the injector temperature was set at 250°C and the detector temperature at 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 based on the retention time of the internal standard free fatty acid C17.

2.4 Tocol Determination. Tocol extraction was based on the alkaline saponification and hexane extraction method used previously for cereals and nuts (Xu 2002). Briefly, almond powder (0.25 g) was mixed with ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% 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, vortexing periodically (every 10 min). The tubes were then placed in ice water for 5 min, water (1.5 mL) and hexane (2.5 mL) were added and vortexed for 30 s, then centrifuged (1000 g at 20°C for 10 min). The hexane layer was transferred to vials and the residue extracted again, before the hexane was evaporated using a nitrogen evaporator (N-EVAP 112) at 45°C, and hexane (1 mL) was added to each vial for HPLC analysis. The analysis protocol referred to Lampi and colleagues (Lamp et al. 2008, Lampi 2011), specifically, the isocratic mobile phase was hexane (with 2% 1,4-dioxane), flow rate 1.0 mL/min, injection volume 20 µL, column temperature 25°C. HPLC analysis was performed using an Agilent 1200 HPLC (Agilent Technologies, Deutschland, Germany) coupled with a diode array detector (DAD), fluorescence detector (FLD), autosampler, quaternary pump, Agilent Chemstation and Grace Alltime HP silica column (150 mm, 3 mm, 3 µm; Grace Discovery Sciences, Deerfield, IL. USA). α, β, γ, δ-Tocopherol and α-tocotrienol were quantified using calibration curves prepared from external standards. α-Tocopherol was measured by DAD at a signal wavelength of 292 nm, while β, γ, δ-tocopherol and α-tocotrienol were analysed by FLD at signal wavelengths of 292 nm (excitation) and 325 nm (emission).

2.5 Data Analysis. Chemical data were analysed by ANOVA using GenStat (14th Edition, VSN International Limited, Herts, UK) and GraphPad Prism 5 (Version 5.01 GraphPad
RESULTS AND DISCUSSION

3.1 Influence of region and genotype on almond lipid content

The lipid content of the almonds studied ranged from 46.1 to 63.5 g/100 g (Table 1). The lowest lipid content (46.1 g/100 g) was observed in Carmel almonds from California, while Nonpareil almonds from Willunga (Australia) contained the highest lipid level (63.5 g/100 g). The lipid concentrations of Australian grown almond samples were 53.1 to 63.5 g/100 g. This was considered representative of the major commercial varieties grown in the four key regions of Australia and also from the breeding selections, which gave a tighter concentration range compared to lipid levels previously reported for Australian almonds, i.e. 35 to 61 g/100 g (Vezvaei and Jackson 1995, Kodad et al. 2011b).

In this study environment appeared to have a significant effect on the lipid content of Nonpareil, Carmel and Guara almonds, with the northern hemisphere grown almonds containing lower lipid levels (between 46.1-51% of kernel weight) than almonds grown in the southern hemisphere (between 53.7-63.5% of kernel weight) (Supplementary Table 1). For example, Californian Nonpareil lipid content (47.1 g/100 g) was lower than the levels reported in an earlier study (Sathe et al. 2008), but similar with a recent study (Yada et al. 2013). However, Somerton and Johnston varieties showed no compositional differences between sites (Supplementary Table 1). In contrast, genotype had a greater influence on almond lipid content. In the Riverland, lipid content varied significantly between genotypes.
(P< 0.05), in particular, for selection No. 23, which contained 12% higher lipid levels than Nonpareil (Table 1).

3.2 Influence of region and genotype on almond fatty acid composition

Unsaturated fatty acids comprised oleic acid (58.1 – 71.3% of total lipids), linoleic acid (15.7 – 29.9% of total lipids), palmitoleic acid (0.20 – 0.62% of total lipids) and vaccenic acid (0.77 – 2.17% of total lipids), which made up more than 90% of the total lipids. Saturated fatty acids, including palmitic acid (5.9 – 7.5% of total lipids), stearic acid (1.0 – 2.4% of total lipids), arachidic acid (0.07 – 0.10% of total lipids) and myristic acid (0.02 – 0.05% of total lipids, data not shown in the table) accounted for the remaining 10%. These results demonstrate the influence of environment and genotype on almond fatty acid composition (Table 1 and Supplementary Table1). The subsequent discussion focuses on oleic acid and linoleic acid, given the importance of these fatty acids.

Oleic acid and linoleic acid were the most abundant fatty acids, in agreement with previous studies on almonds grown around the world, i.e. in Turkey, Iran, Spain, Italy, China, India, California (Kodad et al. 2004, Askin et al. 2007, Kodad and Socias i Company 2008, Socias i Company et al. 2008, Kodad et al. 2010, Moayedi et al. 2011, Tian et al. 2011). A negative correlation was observed between oleic acid and linoleic acid concentrations (Pearson r -0.993, p value (two tails) < 0.0001) as in other studies (Kodad and Socias i Company 2008, Sathe et al. 2008, Kodad et al. 2010, Kodad et al. 2011b,). No correlation was observed between any lipid fractions and the total lipid content not even with the major fatty acid, oleic acid (Table 2), which was also consistent with previous reports (Sathe et al. 2008, Kodad et al. 2011b).

This study has shown that despite oleic acid being the most abundant fatty acid (approx. 60% of total lipid), it was not correlated with total lipid content, which may be due to the strong
negative correlation (Table 2) of oleic acid with the second major fatty acid, linoleic acid (approx. 30% of total lipid).

Linoleic acid concentrations were significantly affected by both environment and genotype (Table 1 and Supplementary Table 1). Willunga grown almonds contained more linoleic acid than almonds from other sites (Supplementary Table 1). For example, Nonpareil almonds grown in Willunga contained significantly higher linoleic acid levels than Nonpareil almonds grown in the Riverland, Sunraysia and Adelaide plains, by 17%, 19% and 20%, respectively. Similarly, Somerton and Johnston almonds grown in Willunga contained 12% and 16% more linoleic acid than Riverland and Adelaide plains almonds, respectively. Considering the environmental influence, solar radiation and temperature (Figures 1 and 2) varied between sites during the 2012 growing season. However, differences observed in rainfall would likely be negated by irrigation. Willunga experienced less solar radiation than the other sites (Figure 1), which may lead to higher linoleic acid content. Willunga also experienced comparatively lower temperatures than the other sites (Figure 2), which could influence linoleic acid concentrations, i.e. high temperatures have been shown to negatively impact linoleic acid synthesis in sunflower seeds (Harris et al. 1978).

Australian grown almonds also had higher linoleic acid levels between 18.1 – 29.9% than Spanish almonds (Table 1). According to the literature, Spanish almonds contain linoleic acid levels ranging from 12.6 – 27.1% (Kodad and Socias I Company 2008), while Mediterranean almonds contained between 12.9 – 25.9% linoleic acid (Kodad et al. 2011b). Californian almonds were reported to contain 21.5 – 31.1% linoleic acid (Sathe et al. 2008). Noticeably, the regions producing almonds with lower linoleic acid are not irrigated, whereas Californian and Australian regions routinely apply irrigation to their orchards. Irrigation could therefore
be a reason for linoleic acid differentiation. Nanos and colleagues found irrigation resulted in lower linoleic acid in a Texas almond variety, but not in a Ferragnès almond variety (Nanos et al. 2002). Apparently, environmental factors such as irrigation, as well as genotype, can influence almond linoleic acid levels.

The effect of genotype on linoleic acid was highly significant at three sites. The linoleic acid content of Nonpareil almonds was higher than for local Johnston, Somerton and Chellaston varieties, but significantly lower than for Carmel, Peerless and Price varieties, as well as for selections No.12, No.31 and No.13 (Table 1). These results might provide an explanation for the popularity of Nonpareil with industry, in addition to its aesthetically pleasing appearance. These new selections might therefore offer a unique marketing perspective, i.e. based on nutritional status.

A negative correlation between oleic acid and linoleic acid was observed (Table 2), and the relationship between these two fatty acids has been demonstrated in many studies (Kodad et al. 2004, Askin et al. 2007, Sathe et al. 2008, Socias i Company et al. 2008, Kodad et al. 2010, Kodad et al. 2011b, Moayedi et al. 2011, Tian et al. 2011, Zaplin et al. 2013). In this study, significant differences were observed in the oleic acid levels of almonds harvested from different genotypes and from different regions. The highest oleic acid level (68.1% of total lipids) was observed in Nonpareil almonds from Spain, followed by Nonpareil almonds (65.6% of total lipids) from California (Supplementary Table 1). Nonpareil almonds produced in Australia generally contained lower oleic acid levels, albeit Nonpareil almonds from Adelaide plains were similar to almonds from California, with 64.2% of total lipids. Likewise, Guara almonds from Spain contained oleic acid levels greater than Guara almonds from Australian Riverland, but the oleic acid content of Carmel almonds showed no significant difference
when grown in California or Australia (Supplementary Table 1). On the other hand, genotype had a significant impact on oleic acid levels for almonds grown at the same sites; i.e. in Willunga, Somerton almonds had the highest oleic acid levels, followed by Johnston and Nonpareil almonds (Table 1). In Adelaide Plains, the local varieties Chellaston and Johnston yielded almonds that also contained more oleic acid than Nonpareil almonds. In the Riverland, Somerton and selection No.23 almonds had the highest oleic acid levels (Table 1). The differentiation of oleic acid and linoleic acid concentrations led to variation in the O/L ratio (oleic/linoleic) for almonds of different varieties and from different regions. We found that the O/L ratio largely depended on linoleic acid rather than oleic acid levels, due to a highly negative relationship between the O/L ratio and linoleic acid (Pearson r 0.9984, 95% CI 0.9974 - 0.9991, p value (two tailed) <0.0001) which is greater than the positive relationship between the O/L ratio and oleic acid (Pearson r 0.9892, 95% CI 0.9819 – 0.9936, p value (two tailed) <0.0001). The O/L ratio typically represents almond kernel shelf-life: with higher O/L ratio indicating greater storage capacity (Maguire et al. 2004, Piscopo et al. 2010, Kodad et al. 2011b). However, a higher O/L ratio means a lower proportion of linoleic acid, i.e. the healthiest fatty acid present in almond lipids. Therefore, consideration of high O/L ratio for long storage and high linoleic acid for more nutrition has opened space to further research.

### 3.3 Influence of variety/region on almond tocols

Almond storage time not only depends on the O/L ratio, but also the relative concentration of Vitamin E compared to almond lipids; since higher Vitamin E content can improve lipid resistance to rancidity (Socias i Company et al. 2010). Therefore, breeding programs aim to improve the O/L ratio and Vitamin E content of almonds. As such, the Vitamin E content of different varieties and selections grown in particular regions was investigated. Vitamin E comprises eight tocopherol homologues: α, β, γ and δ-tocopherol and α, β, γ and δ-tocotrienol. In this study, four homologues α-tocopherol, β-tocopherol, γ-tocopherol and α-tocotrienol,
were quantified in each of the different samples (Table 3). α-Tocopherol was the major component and accounted for more than 90% of the total tocols, followed by γ-tocopherol, in agreement with previous studies (Delgado-Zamarreno et al. 2004, Kodad et al. 2006, Kornsteiner et al. 2006, Kodad et al. 2009, Matthäus and Özcan 2009, Kodad et al. 2011a, Lopez-Ortiz et al 2012). β-Tocopherol was the third most abundant tocol in Australian grown almonds, but not in Spanish or Californian almonds, which instead, contained higher concentrations of α-tocotrienol. δ-Tocopherol was only observed in trace amounts, as reported elsewhere (Delgado-Zamarreno et al. 2004, Kornsteiner et al. 2006, Lopez-Ortiz et al. 2012, Matthäus and Özcan 2009). α-Tocotrienol was detected in all samples, but considerable variation was observed between Australian almonds and almonds grown overseas (Table 3 and Supplementary Table 2). Previous studies by Kodad and colleagues (Kodad et al. 2006, Kodad et al. 2009, Kodad et al. 2011a) and Maguire (Maguire et al. 2004) reported high concentrations of δ-tocopherol, while, Zacheo and colleagues (Zacheo et al. 2000) detected β-tocopherol at concentrations greater than γ-tocopherol in almonds from an Italian growing region. The variation in tocol concentrations observed in almonds from different origins indicated that both genotype and environment likely affect almond tocol composition.

In this study, we found environment significantly influenced α-tocopherol levels in Nonpareil almonds (Supplementary Table 2), but not in almonds of other varieties. Genotype affected α-tocopherol concentrations in Riverland almonds (Table 3, \( p < 0.001 \)). We observed that Guara had the highest α-tocopherol concentration (18.4 mg per 100 g dry weight), followed by selection No. 13 (15.2 mg per 100 g dry weight), selection 31 and Carmel (both 14.4 mg per 100 g dry weight). In contrast, γ-tocopherol concentration was more strongly affected by environment and genotype than α-tocopherol concentration; an environmental effect on γ-tocopherol levels occurred in Nonpareil, Johnston, Carmel and Guara almonds, but not in Somerton almonds (Table 3 and Supplementary Table 2). Variation due to genotype was
observed at all three sites, i.e. Adelaide plains, Willunga and Riverland (Table 3). β-Tocopherol was influenced by environment and genotype, with significant differences observed in all cases (Table 3 and Supplementary Table 2). Noticeably, α-tocotrienol showed little difference among Australian almonds from different growing regions, but varied widely between southern and northern regions: i.e. Spanish and Californian almonds contained higher concentrations of α-tocotrienol than Australian almonds (Table 3 and Supplementary Table 2). The levels of α-tocotrienol observed equaled or surpassed the γ-tocopherol levels of Spanish and Californian almonds (Table 3). This finding has not been reported in previous research. One possible explanation is due to geographical origin, but the exact factors influencing γ-tocopherol remain unclear. Further research is required to investigate the factors affecting tocol composition of almonds from different growing regions.

Intriguingly, we found an environmental influence on almond tocol homologues that may also depend with genotype. For example, a large difference was seen in the α-tocopherol concentration of Nonpareil almonds, with 23% higher levels corresponding to Willunga and Adelaide plains Nonpareil than Riverland and Sunraysia Nonpareil. This could be due to the lower solar radiation experienced in Willunga, compared to the Riverland and Sunraysia (Figure 1). However, differentiation did not occur in other genotypes. In addition, α-tocopherol was quite stable in Carmel almonds grown in distinct regions; Australian Riverland Carmel and Californian Carmel contained 14.4 mg/100 g and 14.6 mg/100 g, respectively. Likewise, Guara almonds had similar β-tocopherol levels when grown in the Australian Riverland or Spain, i.e. 0.08 mg/100 g and 0.10 mg/100 g, respectively (Table 3 and Supplementary Table 2). These results therefore suggest that the environmental influence on almond tocol homologues also depends on the genotype involved.
CONCLUSIONS

In summary, this study compared the influence of genotype and environment on almond lipids, the major unsaturated fatty acids, oleic acid and linoleic acid, and the nutritionally important tocopherols, i.e. almond components with functional antioxidant properties. Australian grown almonds had higher concentrations of linoleic acid than almonds produced in Mediterranean regions, but the results suggested that genetic factors were more likely to affect almond tocopherol concentration than environmental conditions. Selections No. 13 and No. 23 from the Australian Breeding Program were considered to offer unique marketing potential, given both their favourable kernel appearance, Vitamin E content, kernel storage capacity and nutritional status.

SUPPLEMENTARY INFORMATION

Data showing the influences of environmental conditions on almond lipophilic antioxidants are presented in Supplementary Tables 1 and 2.

ACKNOWLEDGEMENTS

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### Table 1. Lipid and Fatty Acid Compositions of Almonds from Different Regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample</th>
<th>Genotype</th>
<th>Lipids (g/100g)</th>
<th>Palmitic (C16:0)</th>
<th>Palmitoleic (C16:1n-7)</th>
<th>Stearic (C18:0)</th>
<th>Vaccenic (C18:1n-7)</th>
<th>Oleic (C18:1n-9)</th>
<th>Linoleic (C18:2n-6)</th>
<th>O/L Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Johnston</td>
<td>60.8 ± 0.8</td>
<td>6.9 ± 0.0 a</td>
<td>0.44 ± 0.00 a</td>
<td>2.0 ± 0.0 b</td>
<td>1.1 ± 0.0 b</td>
<td>63.0 ± 0.1 b</td>
<td>25.0 ± 0.1 b</td>
<td>2.52 ± 0.01 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonpareil</td>
<td>63.5 ± 0.7</td>
<td>6.8 ± 0.0 a</td>
<td>0.41 ± 0.01 b</td>
<td>1.4 ± 0.1 c</td>
<td>1.2 ± 0.0 a</td>
<td>58.5 ± 0.4 c</td>
<td>29.9 ± 0.6 a</td>
<td>1.96 ± 0.04 c</td>
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<td></td>
<td></td>
<td>Somerton</td>
<td>60.5 ± 1.5</td>
<td>6.1 ± 0.0 b</td>
<td>0.41 ± 0.00 b</td>
<td>2.4 ± 0.0 a</td>
<td>1.0 ± 0.0 c</td>
<td>67.0 ± 0.1 a</td>
<td>21.4 ± 0.2 c</td>
<td>3.13 ± 0.02 a</td>
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<td>Chellaston</td>
<td>58.3 ± 1.1</td>
<td>6.8 ± 0.0</td>
<td>0.44 ± 0.01 b</td>
<td>2.0 ± 0.0 a</td>
<td>1.2 ± 0.0 b</td>
<td>66.6 ± 0.4 a</td>
<td>21.3 ± 0.5 b</td>
<td>3.13 ± 0.06 a</td>
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<tr>
<td></td>
<td></td>
<td>Johnston</td>
<td>58.2 ± 0.7</td>
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<td>0.56 ± 0.01 a</td>
<td>1.8 ± 0.1 b</td>
<td>1.3 ± 0.0 a</td>
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<td>21.0 ± 0.6 b</td>
<td>3.19 ± 0.07 a</td>
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<td>Nonpareil</td>
<td>60.0 ± 1.1</td>
<td>6.9 ± 0.1</td>
<td>0.49 ± 0.01 b</td>
<td>1.4 ± 0.0 c</td>
<td>1.3 ± 0.0 a</td>
<td>64.2 ± 0.4 b</td>
<td>23.9 ± 0.5 a</td>
<td>2.69 ± 0.04 b</td>
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<tr>
<td>Riverland</td>
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<td>Carmel</td>
<td>53.7 ± 1.0 b</td>
<td>6.8± 0.0 b</td>
<td>0.39 ± 0.01 cd</td>
<td>1.2 ± 0.0 d</td>
<td>1.2 ± 0.0 f</td>
<td>63.0 ± 0.4 b</td>
<td>29.8 ± 0.5 a</td>
<td>1.96 ± 0.03 i</td>
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<td></td>
<td></td>
<td>Guara</td>
<td>60.4 ± 2.0 ab</td>
<td>5.9 ± 0.0 e</td>
<td>0.23 ± 0.00 f</td>
<td>2.4 ± 0.0 a</td>
<td>0.8 ± 0.0 h</td>
<td>66.4 ± 0.0 d</td>
<td>22.3 ± 0.1 f</td>
<td>2.97 ± 0.01 e</td>
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<td></td>
<td>Nonpareil</td>
<td>56.5 ± 2.8 b</td>
<td>6.7 ± 0.1 c</td>
<td>0.45 ± 0.02 b</td>
<td>1.3 ± 0.0 cd</td>
<td>1.3 ± 0.0 e</td>
<td>63.6 ± 0.7 d</td>
<td>24.8 ± 0.9 e</td>
<td>2.57 ± 0.08 f</td>
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<td></td>
<td>Peerless</td>
<td>54.5 ± 1.0 b</td>
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<td>0.39 ± 0.00 c</td>
<td>1.1 ± 0.0 de</td>
<td>1.3 ± 0.0 c</td>
<td>61.1 ± 0.1 d</td>
<td>27.3 ± 0.2 c</td>
<td>2.24 ± 0.01 h</td>
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<td></td>
<td>Price</td>
<td>53.1 ± 1.5 b</td>
<td>6.2 ± 0.0 de</td>
<td>0.31 ± 0.01 f</td>
<td>1.3 ± 0.0 cd</td>
<td>1.2 ± 0.0 g</td>
<td>63.2 ± 0.3 d</td>
<td>25.9 ± 0.4 d</td>
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<tr>
<td></td>
<td></td>
<td>SMT</td>
<td>59.4 ± 0.8 ab</td>
<td>5.9 ± 0.0 e</td>
<td>0.40 ± 0.00 bc</td>
<td>1.7 ± 0.0 b</td>
<td>1.2 ± 0.0 g</td>
<td>70.1 ± 0.2 a</td>
<td>18.9 ± 0.2 h</td>
<td>3.71 ± 0.03 b</td>
</tr>
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<td>54.7 ± 2.6 b</td>
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<td>0.48 ± 0.00 a</td>
<td>1.1 ± 0.0 de</td>
<td>1.4 ± 0.0 b</td>
<td>67.5 ± 0.2 c</td>
<td>21.0 ± 0.2 g</td>
<td>3.22 ± 0.03 d</td>
</tr>
<tr>
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<td>57.4 ± 2.5 b</td>
<td>6.8 ± 0.0 bc</td>
<td>0.35 ± 0.00 e</td>
<td>1.0 ± 0.0 e</td>
<td>1.3 ± 0.0 e</td>
<td>62.3 ± 0.1 d</td>
<td>26.3 ± 0.2 d</td>
<td>2.37 ± 0.02 g</td>
</tr>
<tr>
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<td>12</td>
<td>61.3 ± 3.1 ab</td>
<td>7.4 ± 0.2 a</td>
<td>0.48 ± 0.01 a</td>
<td>1.3 ± 0.0 c</td>
<td>1.5 ± 0.0 a</td>
<td>59.3 ± 0.2 d</td>
<td>28.2 ± 0.4 b</td>
<td>2.11 ± 0.03 i</td>
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<td>63.3 ± 0.9 a</td>
<td>6.6 ± 0.0 c</td>
<td>0.37 ± 0.01 d</td>
<td>1.3 ± 0.0 cd</td>
<td>1.1 ± 0.0 h</td>
<td>70.8 ± 0.2 a</td>
<td>18.1 ± 0.3 h</td>
<td>3.92 ± 0.05 a</td>
</tr>
<tr>
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<td>31</td>
<td>62.4 ± 1.4 ab</td>
<td>7.5 ± 0.0 a</td>
<td>0.46 ± 0.00 ab</td>
<td>1.4 ± 0.0 c</td>
<td>1.2 ± 0.0 f</td>
<td>59.9 ± 0.4 d</td>
<td>27.6 ± 0.5 bc</td>
<td>2.17 ± 0.04 hi</td>
</tr>
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<td></td>
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<td>32</td>
<td>53.3 ± 0.5 b</td>
<td>6.2 ± 0.0 e</td>
<td>0.33± 0.01 e</td>
<td>1.8 ± 0.1 b</td>
<td>1.1 ± 0.0 h</td>
<td>67.0 ± 0.5 cd</td>
<td>21.5 ± 0.8 g</td>
<td>3.12 ± 0.09 ed</td>
</tr>
<tr>
<td></td>
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<td>48</td>
<td>58.4 ± 1.9 ab</td>
<td>6.4 ± 0.0 d</td>
<td>0.47 ± 0.01 a</td>
<td>1.2 ± 0.0 cd</td>
<td>1.3 ± 0.0 d</td>
<td>68.8 ± 0.3 b</td>
<td>19.9 ± 0.4 h</td>
<td>3.45 ± 0.05 c</td>
</tr>
</tbody>
</table>

Notes: a, b, c, d, e, f, g, h, i denote differences at 0.01, 0.05, 0.001, and <0.001 levels of significance, respectively.
Table 1. Lipid and Fatty Acid Compositions of Almonds from Different Regions (cont.)

<table>
<thead>
<tr>
<th>Region</th>
<th>Nonpareil</th>
<th>Carmel</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>47.1 ± 3.2</td>
<td>46.1 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>6.6 ± 0.0</td>
<td>7.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>0.48 ± 0.01</td>
<td>0.44 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.2 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1.5 ± 0.0</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>65.6 ± 0.1</td>
<td>58.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>22.6 ± 0.0</td>
<td>29.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2.90 ± 0.01</td>
<td>1.96 ± 0.00</td>
</tr>
</tbody>
</table>

Values are means of 3 replicates ± standard error, followed by different letters within a column are significantly different ($P < 0.05$); ns = not significant. *Fatty acid content as a percentage of lipids.

Table 2. Correlation Matrix for Almond Lipids and Fatty Acids

<table>
<thead>
<tr>
<th></th>
<th>lipids</th>
<th>palmitic (C16:0)</th>
<th>palmitoleic (C16:1n-7)</th>
<th>stearic (C18:0)</th>
<th>vaccenic (C18:1n-7)</th>
<th>oleic (C18:1n-9)</th>
<th>linoleic (C18:2n-6)</th>
<th>arachidic (C20:0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lipids</td>
<td>0.202</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>palmitic</td>
<td>0.173</td>
<td>0.612**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>palmitoleic</td>
<td>0.315</td>
<td>-0.500*</td>
<td>-0.393</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stearic</td>
<td>-0.204</td>
<td>0.625**</td>
<td>0.758***</td>
<td>-0.754***</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vaccenic</td>
<td>0.023</td>
<td>-0.634**</td>
<td>-0.048</td>
<td>0.327</td>
<td>-0.280</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>oleic</td>
<td>-0.065</td>
<td>0.566**</td>
<td>-0.021</td>
<td>-0.355</td>
<td>0.251</td>
<td>-0.993***</td>
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<tr>
<td>linoleic</td>
<td>0.229</td>
<td>-0.126</td>
<td>-0.025</td>
<td>0.477*</td>
<td>-0.134</td>
<td>-0.019</td>
<td>-0.012</td>
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<tr>
<td>arachidic</td>
<td>0.229</td>
<td>-0.126</td>
<td>-0.025</td>
<td>0.477*</td>
<td>-0.134</td>
<td>-0.019</td>
<td>-0.012</td>
<td>-</td>
</tr>
</tbody>
</table>

Pearson r values indicates significant correlations (CI 95%, * denotes $P \leq 0.05$, ** denotes $P \leq 0.005$, *** denotes $P \leq 0.001$).
Table 3. Tocol Composition of Almonds from Different Regions

<table>
<thead>
<tr>
<th>region</th>
<th>genotype</th>
<th>total tocols</th>
<th>α-tocopherol</th>
<th>γ-tocopherol</th>
<th>β-tocopherol</th>
<th>α-tocotrienol</th>
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<tbody>
<tr>
<td></td>
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<td>concentration (mg/100 g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>region</td>
<td>genotype</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Willunga</td>
<td>Johnston</td>
<td>11.8 ± 1.8 a</td>
<td>11.5 ± 1.7 a</td>
<td>0.3 ± 0.0 b</td>
<td>0.04 ± 0.00 b</td>
<td>0.01 ± 0.00 c</td>
</tr>
<tr>
<td></td>
<td>Nonpareil</td>
<td>14.4 ± 0.5 a</td>
<td>13.7 ± 0.5 a</td>
<td>0.6 ± 0.0 a</td>
<td>0.09 ± 0.01 a</td>
<td>0.05 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Somerton</td>
<td>9.5 ± 0.1 b</td>
<td>9.3 ± 0.1 b</td>
<td>0.2 ± 0.0 b</td>
<td>0.04 ± 0.00 b</td>
<td>0.04 ± 0.00 b</td>
</tr>
<tr>
<td></td>
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<td>P</td>
<td>&lt; 0.05</td>
<td>ns</td>
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<td>&lt; 0.001</td>
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<td>Adelaide</td>
<td>Chellaston</td>
<td>10.4 ± 1.7 a</td>
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<td>0.2 ± 0.0 ab</td>
<td>0.05 ± 0.00 b</td>
<td>0.04 ± 0.01 a</td>
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<td>9.8 ± 0.2 a</td>
<td>0.2 ± 0.0 b</td>
<td>0.07 ± 0.00 a</td>
<td>0.01 ± 0.00 b</td>
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<tr>
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<td>Nonpareil</td>
<td>14.4 ± 1.3 a</td>
<td>13.9 ± 1.2 a</td>
<td>0.3 ± 0.0 a</td>
<td>0.08 ± 0.00 a</td>
<td>0.04 ± 0.00 a</td>
</tr>
<tr>
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<td>P</td>
<td>ns</td>
<td>ns</td>
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<td>&lt; 0.05</td>
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<td>Carmel</td>
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<td>14.4 ± 0.8 b</td>
<td>0.2 ± 0.0 b</td>
<td>0.07 ± 0.00 e</td>
<td>0.02 ± 0.00 d</td>
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<tr>
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<td>Guara</td>
<td>18.9 ± 1.2 a</td>
<td>18.4 ± 1.1 a</td>
<td>0.4 ± 0.0 a</td>
<td>0.08 ± 0.00 d</td>
<td>0.09 ± 0.01 a</td>
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<td>11.0 ± 0.4 cd</td>
<td>10.7 ± 0.4 cd</td>
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<td>0.06 ± 0.01 ef</td>
<td>0.02 ± 0.00 d</td>
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<td>13.8 ± 1.0 bc</td>
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<td>0.02 ± 0.00 d</td>
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<td>0.2 ± 0.0 c</td>
<td>0.09 ± 0.00 c</td>
<td>0.02 ± 0.00 d</td>
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<tr>
<td></td>
<td>Somerton</td>
<td>12.1 ± 1.3 c</td>
<td>11.8 ± 1.3 c</td>
<td>0.2 ± 0.0 bc</td>
<td>0.06 ± 0.00 f</td>
<td>0.03 ± 0.00 cd</td>
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<td>0.11 ± 0.00 ab</td>
<td>0.03 ± 0.00 cd</td>
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<td>0.04 ± 0.00 b</td>
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<td>9.2 ± 0.1 d</td>
<td>0.2 ± 0.0 c</td>
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<td>0.02 ± 0.00 d</td>
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<td>0.05 ± 0.00 f</td>
<td>0.02 ± 0.00 d</td>
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<td>0.4 ± 0.0 a</td>
<td>0.06 ± 0.00 f</td>
<td>0.03 ± 0.00 cd</td>
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<td>No. 32</td>
<td>10.6 ± 0.7 cd</td>
<td>10.5 ± 0.7 cd</td>
<td>0.1 ± 0.0 d</td>
<td>0.05 ± 0.00 f</td>
<td>0.03 ± 0.00 cd</td>
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<td>11.4 ± 0.9 c</td>
<td>0.2 ± 0.0 bc</td>
<td>0.07 ± 0.00 ef</td>
<td>0.03 ± 0.00 c</td>
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<td>&lt; 0.001</td>
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<td>&lt; 0.001</td>
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<td>10.7 ± 0.8</td>
<td>0.2 ± 0.0</td>
<td>0.07 ± 0.00</td>
<td>0.03 ± 0.00</td>
</tr>
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<td>Variety</td>
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<td>------------</td>
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</tr>
<tr>
<td>Spain</td>
<td>Nonpareil</td>
<td>18.5 ± 1.7</td>
<td>17.9 ± 1.7</td>
<td>0.3 ± 0.0</td>
<td>0.09 ± 0.01</td>
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</tr>
<tr>
<td></td>
<td>Guara</td>
<td>21.5 ± 0.9</td>
<td>20.9 ± 0.9</td>
<td>0.3 ± 0.0</td>
<td>0.12 ± 0.01</td>
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</tr>
<tr>
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<td>Nonpareil</td>
<td>18.3 ± 1.6</td>
<td>17.9 ± 1.6</td>
<td>0.1 ± 0.0</td>
<td>0.01 ± 0.00</td>
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</tr>
<tr>
<td></td>
<td>Carmel</td>
<td>14.9 ± 1.7</td>
<td>14.6 ± 1.7</td>
<td>0.1 ± 0.0</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
</tbody>
</table>

Values are means of three replicates ± standard error, followed by different letters within a column are significantly different (P < 0.05); ns = not significant.
Figure 3. Monthly mean solar exposure (MJ/m$^2$) during the 2011/2012 almond growing season.

Figure 4. Monthly mean maximum and minimum temperature (°C) during the 2011/12 almond growing season.

Table 4. Supplementary Table 1. Lipid and Fatty Acid Compositions of Almonds from Different Genotypes

<table>
<thead>
<tr>
<th>sample</th>
<th>genotype</th>
<th>region</th>
<th>lipids (g/100g)</th>
<th>palmitic (C16:0)</th>
<th>palmitoleic (C16:1n-7)</th>
<th>stearic (C18:0)</th>
<th>vaccenic (C18:1n-7)</th>
<th>oleic (C18:1n-9)</th>
<th>linoleic (C18:2n-6)</th>
<th>ratio O/L</th>
</tr>
</thead>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Willunga</td>
<td></td>
<td>63.5 ± 0.7 a</td>
<td>6.8 ± 0.0 c</td>
<td>0.41 ± 0.01 d</td>
<td>1.4 ± 0.1 a</td>
<td>1.2 ± 0.0 d</td>
<td>58.5 ± 0.4 d</td>
<td>29.9 ± 0.6 a</td>
<td>1.96 ± 0.04 d</td>
</tr>
<tr>
<td></td>
<td>Adelaide</td>
<td></td>
<td>60.0 ± 1.1 ab</td>
<td>6.9 ± 0.1 bc</td>
<td>0.49 ± 0.01 c</td>
<td>1.4 ± 0.0 a</td>
<td>1.3 ± 0.0 c</td>
<td>64.2 ± 0.4 bc</td>
<td>23.9 ± 0.5 c</td>
<td>2.69 ± 0.04 c</td>
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<td>Riverland</td>
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<td>1.3 ± 0.0 b</td>
<td>1.3 ± 0.0 c</td>
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<td>2.57 ± 0.08 c</td>
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<td>6.9 ± 0.0 b</td>
<td>0.52 ± 0.01 b</td>
<td>1.1 ± 0.0 c</td>
<td>1.5 ± 0.0 b</td>
<td>63.8 ± 0.3 c</td>
<td>24.4 ± 0.4 bc</td>
<td>2.61 ± 0.03 c</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td></td>
<td>51.0 ± 2.0 bc</td>
<td>7.5 ± 0.0 a</td>
<td>0.62 ± 0.00 a</td>
<td>1.4 ± 0.0 ab</td>
<td>2.2 ± 0.0 a</td>
<td>68.1 ± 0.1 a</td>
<td>19.0 ± 0.0 e</td>
<td>3.58 ± 0.00 a</td>
</tr>
<tr>
<td></td>
<td>California</td>
<td></td>
<td>47.1 ± 3.2 c</td>
<td>6.6 ± 0.0 c</td>
<td>0.48 ± 0.01 cd</td>
<td>1.2 ± 0.0 b</td>
<td>1.5 ± 0.0 b</td>
<td>65.6 ± 0.1 b</td>
<td>22.6 ± 0.0 d</td>
<td>2.90 ± 0.01 b</td>
</tr>
<tr>
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<td></td>
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<td>0.41 ± 0.00</td>
<td>2.4 ± 0.0 a</td>
<td>1.0 ± 0.0 b</td>
<td>67.0 ± 0.1 a</td>
<td>21.4 ± 0.2 a</td>
<td>3.13 ± 0.02 b</td>
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<tr>
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<td></td>
<td>59.4 ± 0.8</td>
<td>5.9 ± 0.0 b</td>
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<td>2.0 ± 0.0 a</td>
<td>1.1 ± 0.0 b</td>
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Values are means of three replicates ± standard error. Values followed by different letters within a column are significantly different (\( P < 0.05 \)); ns = not significant.

*Fatty acid content as a percentage of lipids.
Table 5. Supplementary Table 2. Tocol Composition of Almonds from Different Genotypes

<table>
<thead>
<tr>
<th>genotype</th>
<th>site</th>
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<th>total tocols</th>
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<th>γ-tocopherol</th>
<th>β-tocopherol</th>
<th>α-tocotrienol</th>
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<td>18.5 ± 1.7 a</td>
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<tr>
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Values are means of three replicates ± standard error. Values followed by different letters within a column are significantly different (P < 0.05); ns = not significant.
CHAPTER THREE

Influence of Deficit Irrigation Strategies on Fatty Acid and Tocopherol Concentration of Almond

Introduction

Australia is the major almond producer in the southern hemisphere (Wilkinson 2012b). Within Australia, almond growing regions are located: along the corridor of Murray River, between Renmark in South Australia and Sunraysia in Victoria and in the Riverina of New South Wales (Almond Board of Australia 2012a). These regions typically experience Mediterranean climates that are ideally suited for almond production, albeit the hot and dry conditions during most summers, necessitates irrigation of almond orchards (Goldhamer et al. 2006, Kodad et al. 2010, Wilkinson 2012b).

In 2009, the Australian almond industry, in collaboration with the funding body Horticulture Australia Limited (HAL) and the Department of Environment and Primary Industry Victoria (DEPI Vic.), established a project titled ‘Optimising water use of Australian almond production through deficit irrigation strategies’, to investigate the yield responses to deficit irrigation and to benchmark efficient water use in almond production.

The research outlined in the following paper sought to extend the above project, by investigating the impact of deficit irrigation strategies on the fatty acid and tocopherol concentration of almonds. Since the concentrations of fatty acids and tocopherols strongly influence almond quality, it is important to understand the impact of irrigation management strategies on almond composition, in addition to plant physiological responses, such as crop yield.
Paper: Effects of Drought Stress On Fatty Acid and Tocopherol Concentration of Almonds (*Prunus dulcis*)

Ying Zhu\(^{(1)}\), Cathy Taylor\(^{(2)}\), Karl Sommer\(^{(2)}\), Kerry Wilkinson\(^{(1)}\), Michelle Wirthensohn\(^{(1)}\)*

1. University of Adelaide, School of Agriculture, Food and Wine, PMB 1, SA 5064
2. Department of Environment and Primary Industries, Future Farming Systems Research Division, PO Box 905, Mildura, VIC 3502

*Email for Correspondence: michelle.wirthensohn@adelaide.edu.au

(Planned to submit to the Journal of Agricultural Water Management)
ABSTRACT

The effects of deficit irrigation on almond fatty acid and tocopherol levels were studied in a field trial. Mature almond trees were subjected to three levels of deficit irrigation (85%, 70% and 55% of potential crop evapotranspiration ET₀), as well as control (100% ET₀) and over-irrigation (120% ET₀) treatments. Two deficit irrigation strategies were employed: regulated deficit irrigation (RDI) and sustained deficit irrigation (SDI). Compositional analysis of almonds harvested from two seasons showed moderate deficit irrigation (85% RDI and 85% SDI) had no detrimental impact on almond kernel lipid content, but severe and extreme deficiencies (70% and 55%) influenced lipid content. Unsaturated fatty acid (USFA) and saturated fatty acid (SFA) contents fluctuated under these treatments, the oleic/linoleic ratio increased under moderate water deficiency, but decreased under severe and extreme water deficiency. γ-Tocopherol levels were relatively stable under deficit irrigation and α, β-tocopherol and α-tocotrienol were not affected. The variation between years indicated climate has an effect on almond fruit development. In conclusion it is feasible to irrigate almond trees using less water than the normal requirement, without the loss of kernel nutrition quality.

KEY-WORDS: water stress, tocopherols, lipids, fatty acids, Prunus dulcis, RDI, SDI
INTRODUCTION

Drought is an agronomic issue of growing importance and one of the abiotic stresses, for which plants have developed various adaptation strategies (Ramanjuju and Bartel 2002; Chaves et al. 2003). Many studies have investigated the impact of drought on plant physiology and/or crop yield by studying model plants in the laboratory to explore genes associated with drought resistance tolerance (Gigon et al. 2004; Tang et al. 2011), or in the field to determine the physical and chemical changes over a number of plants (Hamrouni et al. 2001; Baldini et al. 2002; Karam et al. 2007; Laribi et al. 2009; Bettaieb et al. 2012). Lipids are important indicators of drought stress which can influence leaf cell membrane integrity, or indicate oil crop yield such as soybean, canola, sunflower, sesame and peanut. For example, some studies (Haroumi et al. 2001; Jordan et al. 2006; Petropoulos et al. 2008; Bettaieb et al. 2012) examined leaf lipid composition which could be used for further genetic study, and other studies (Dwivedi et al. 1996; Brits and Kremer 2002; Karam et al. 2007; Ucan et al. 2007; Zarei et al. 2010; Candogan et al. 2013) focused on establishing whether or not water saving solutions were feasible, and could be applied to improve agricultural water management.

Almonds are not considered a practical crop for oil production, but rather a lipid-rich food source offering health benefits that have been demonstrated in various studies (Spiller et al. 1998; Jia et al. 2006; Hollis and Mattes 2007; Jenkins et al. 2008; Rajaram et al. 2010; Wien et al. 2010; Damasceno et al. 2011). Given almond nutrition largely relies on lipid quality, any water saving solutions employed during almond orchard management, need to consider any effects on almond fatty acid composition and tocopherol concentration that impact lipid quality. This study investigated whether water deficiency during the growing season had any influence (positive or negative) on almond lipid and tocopherol concentration.
The study was part of a wider research project that investigated almond yield responses to deficit irrigation with the aim of establishing water saving benchmarks for almond orchards located in inland Australia with climatic conditions similar to those in the Mediterranean, i.e. comprising hot summers and low precipitation. Irrigation is a priority of almond orchard management, thus, water deficiency irrigation strategies were compared: sustained deficit irrigation (SDI) and regulated deficit irrigation (RDI). SDI and RDI have been studied in Californian almond production (Goldhamer et al. 2006), where climate conditions are also similar to the Mediterranean. In these studies, water was applied to almond orchards either periodically (i.e. RDI) or continuously (i.e. SDI), in order to identify the most effective irrigation method (Goldhamer et al. 2006). Almond biomass and kernel yield were investigated (Goldhamer et al. 2006), but almond lipid quality in particular lipid content, fatty acid composition and tocopherol concentration were not considered. Lipids and tocopherols contribute almond functional properties (Jenkins et al. 2008; Rajaram et al. 2010; Wien et al. 2010; Damasceno et al. 2011), so the impact of orchard management practices on almond nutritional profile should consider not just kernel yield. This project involved a field trial based on a modified method of Goldhamer et al. (2006) to study Australian climate and soil conditions and specifically examined almond lipid content, fatty acid content, tocopherol composition to assess the impact of deficit irrigation strategies on almond lipid quality.

MATERIALS AND METHODS

2.1 Plant Materials

Seven-year-old almond trees (Prunus dulcis, cv. Nonpareil) were subjected to deficit irrigation during the 2009-2012 growing seasons in a field trial conducted at Lake Powell,
Victoria (34.71° S, 142.87° E, elevation 53 m). The design of the field trial was adapted from that of Goldhamer et al (2006) and comprised the following treatments: control (100% normal irrigation requirements, based on crop evapotranspiration ET$_o$), wet (20% over-irrigated) and 3 levels of deficit irrigation being 85%, 70% and 55% ET$_o$. Each deficit irrigation level was achieved via two strategies, i.e. RDI and SDI. SDI was imposed on trees throughout the whole irrigation season i.e. from early August to late May, while RDI was only applied for designated periods (Table 1). Each treatment plot was replicated six times and each plot contained four trees. At commercial maturity, six fruits were randomly harvested from each tree in each plot, and the 24 fruits per plot were stored at 4°C prior to analysis. Randomly, six almond kernels from each group of 24 kernels were randomly subsampled for chemical analysis, giving 6 kernel x 6 replicate equalling 36 kernels in total for chemical analysis at maturity from each of two harvest years, 2010/11 and 2011/12.

2.2 Climate measurement

Climate data (Table 2) were sourced from an automatic weather station (MEA Adelaide) maintained by Lower Murray Water, situated within a few hundred metres of the trial site.

2.3 Chemical Reagents

Hexane, ethanol, methanol, chloroform, n-heptane, sodium chloride, butylatedhydroxyanisole (BHA), sulphuric acid, ascorbic acid, potassium hydroxide were purchased from Merck, Scharlau and Sigma. C17 free fatty acid (C17 FFA from Nucheck Prep Inc.) was used as an internal standard to quantify the fatty acid percentage of almond lipids. For tocopherol identification and quantification, an α, β, γ, δ-tocopherol standard set (Calbiochem, Germany)
and an α-tocotrienol standard (Cayman Chemicals, USA) were used to develop external standard curves.

2.4 Sample Preparation

Kernels were weighed before processing to determine the kernel size. Thereafter, the kernels were dried at 50°C for 48 hours, ground using a coffee grinder to a fine powder and sieved through a 1000 µm mesh, and then stored under nitrogen in glass vials.

2.5 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): sampling almond powder (0.05 g), then the proportional agents used were 0.9% aqueous sodium chloride (2 mL), methanol (3 mL containing 0.005% BHA), free fatty acid C17 (400 µL, 0.16% in methanol) as an internal standard and chloroform (6 mL), the methylation agents were chloroform:methanol (9:1 v/v, 1 mL; containing 0.005% BHA) and methanol (5 mL containing 1% sulphuric acid). The final extract solvents were n-heptane (2 mL) and water (0.75 mL). The extraction procedure followed that of Makrides et al. (1996). The gas chromatography protocol for fatty acid determination was carried out with helium as the carrier gas and 20:1 split-ratio, the injector temperature at 250°C and the detector temperature at 300°C, the initial oven temperature at 140°C increasing to 220°C at 5°C/min, and then held at this temperature for 3 min. FAMEs were identified based on the retention time of the internal standard free fatty acid C17. An HP 6890 Gas Chromatograph (Hewlett Packard, Palo Alto, CA. USA) equipped with a flame ionization detector (FID), HP 7683 autosampler, HP Chemstation software, split/splitless injection and a capillary GC column
SGE BPX 70 (50 m, 0.32 mm ID, 0.25 µm) (SGE Analytical Science Pty. Ltd. Ringwood, Victoria Australia) were used for analysis.

2.6 Tocol Determination

Tocol extraction was based on the alkaline saponification and hexane extraction method used previously for cereals and nuts (Xu, 2002): sampling almond powder (0.25 g) and the following agents were employed proportionally with the sampling amount: ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% 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, vortexing periodically (every 10 min). The tubes were then placed in ice water for 5 min, water (1.5 mL) and hexane (2.5 mL) were added and vortexed for 30 s, then centrifuged (1000 g at 20°C for 10 min). The hexane layer was transferred to vials and the residue extracted again, before the hexane was evaporated using a nitrogen evaporator (N-EVAP 112) at 45°C, and hexane (1 mL) was added to each vial for HPLC analysis. The protocol of HPLC tocopherol qualification and quantification followed that of Lampi et al. (2008, 2011): hexane (with 2% 1,4-dioxane) as the isocratic mobile phase, flow rate 1.0 mL/min, injection volume 20 µL, column temperature 25°C. The analysis was performed under an Agilent 1200 HPLC (Agilent Technologies, Deutschland, Germany) coupled with a diode array detector (DAD), fluorescence detector (FLD), autosampler, quaternary pump, Agilent Chemstation and Grace Alltime HP Silica column (150 mm, 3 mm, 3 µm) (Grace Discovery Sciences, Deerfield, IL. USA). α, β, γ, δ-Tocopherol and α-tocotrienol individual external standards were used for the calibration curves. α-Tocopherol was determined at DAD signal wavelength 292 nm, while β, γ, δ-tocopherol and α-tocotrienol were determined at FLD signal wavelength 292 nm (excitation) and 325 nm (emission).
2.7 Data Analysis

Chemical data were analysed by ANOVA using GenStat (14th Edition, VSN International Limited, Herts UK). Mean comparisons were performed by least significant difference (LSD) multiple-comparison test at p < 0.05.

RESULTS AND DISCUSSION

3.1 Effect of deficit irrigation on almond lipid content

The climatic conditions during the two years were extremely variable and likely had an influence on kernel quality. The 2010/11 season had above average rainfall and was more humid but milder than the 2011/12 season, in particular during the kernel development stages (Table 2). The repeated and heavy rainfall in 2010/11 resulted in a lack of plant water stress despite the imposed irrigation deficits. Figure 1 shows the midday stem water potential which was measured fortnightly during the 2010/11 and 2011/12 growing season and is an accurate indicator of tree water stress. Regardless of treatment, trees were consistently less stressed (less negative values), in the 2011/12 season with above average rainfall than in the preceding 2010/11 season. Therefore, in 2011/12 the deficiency treatments were rendered mostly ineffective and no significant difference in the lipid content between the treatments was observed (Table 3). The climate conditions experienced in the 2011/12 season were more typical (Table 2) and resulted in water stress in line with the imposed deficit treatments. Moderate water deficiency (85% ET₀ both RDI and SDI) in 2011/12 did not affect lipid content compared with the control. Severe and extreme water deficiency (70% and 55% ET₀) decreased almond lipid content below that of the control, but 55% SDI and the wet treatment did not (Table 3). Moreover, two-way ANOVA showed the lipid contents of control, wet and 85% RDI in 2010/11 were significantly lower than the equivalent treatments in 2011/12, and the overall value in 2010/11 was lower by 5.4% than that in 2011/12. It would appear that the
humid but mild growing conditions of 2010/11, relative to the subsequent drier and hotter season, depressed almond kernel lipid synthesis regardless of the imposed deficit treatment. In an average season like that of 2011/12, with generally higher lipid content, moderate water deficiency was not detrimental to almond lipid content but severe deficit irrigation was.

Some studies investigating soybean (Candogan et al. 2013) and sunflower (Baldini et al. 2002) reported a negative relationship between the degree of plant water deficiency and oil content. Conversely our study showed no clear correlation between almond lipid content and the deficiency gradient because of the contrasting weather from season to season. Our observations agree with those of Nanos et al. (2002) and Sanchez-Bel et al. (2008) who investigated the influence of irrigation and harvest time or irrigation and fertilization on the oil content of kernels harvested from various almond cultivars. They all reported that there was no significant difference in the oil content between irrigated and non-irrigated almond trees. Egea et al. (2009) also demonstrated that partial root zone drying did not negatively affect almond lipid content. That soybean and sunflower are annuals might mean they are more susceptible to environmental changes than almond, a perennial plant, with a more extensive root and trunk system and thus a greater resilience to seasonal changes in climate environment. Seasonal weather appeared to affect almond lipid content. The 2011/12 season with above average rainfall and moderate temperature had lower almond lipid content relative to the preceding season which was hotter and drier but was more representative of the long term average weather (Table 1, 2). Moreover, our study showed that the extreme deficiency 55% SDI in 2011/12 did not lower the lipid content relative to control trees. It is possible the trees in 55% SDI treatment were able to better adapt to the imposed drought stress compared to 55% RDI because the SDI treatment was imposed through the whole irrigation season while RDI was only imposed at specific periods during fruit and kernel development (Table
1). It is conceivable that trees adapt better to stable rather than fluctuating stress (Chaves et al. 2003; Goldhamer et al. 2006). Our results suggest that SDI was a more reliable irrigation strategy than RDI.

### 3.2 Change in fatty acid composition under deficit irrigation

In 2010/11, some statistically significant differences in fatty acid composition were observed between treatments (Table 3). However, differences were quite small and may not reflect water deficiency because of the apparent lack of water stress due to the unusually wet season. In 2011/12, when stress was apparent, oleic acid content tended to decrease with greater water deficiency, while the opposite trend was seen with linoleic acid and the O/L ratio (oleic/linoleic) (Table 3). Such differences could be attributed to the water deficiency, but could also be caused by other influences in the field, given the differences seen in the first season (2010/11) when there was no effective water deficiency because of heavy rainfall.

Based on the data obtained in the more typical year 2011/12, the percentage of oleic acid resulting from the 85% RDI treatment was significantly higher than for the control (P<0.001). This appears to be in contrast to Nanos et al. (2002) study where oleic acid levels were higher in irrigated than non-irrigated almonds (cv. Ferragnès and Texas). However, a further rise in the imposed deficit did clearly reduce oleic acid percentage below the control, a result consistent with Nanos et al. (2002). According to the 2011/12 data, moderate water deficiency did not reduce the O/L ratio, rather, it slightly increased under 85% RDI and was similar to the control under 85% SDI (Table 3), but severe and extreme water deficiency caused a lowering of the O/L ratio. Given the O/L ratio is an indicator of almond kernel storage capability and its decline infers shorter kernel storage life (Piscopo et al. 2010), the results
indicated that moderate water deficiency did not impact kernel storage capability, while severe deficiency would shorten kernels storage. Generally, none of the major fatty acids of almond, whether SFA like palmitic and stearic acids or USFA, like oleic and linoleic acids, were resistant to water stress. However, moderate deficiency did not negatively impact almond lipid composition and nutritional value.

3.3 Response of kernel tocopherol concentration to deficit irrigation

The total tocopherol concentration of almonds harvested in 2010/11 was much higher and more variable between treatments than those harvested in 2011/12 (Table 4). The weather in 2010/11 was more humid and milder than in the subsequent season (Table 2) suggesting conditions in the first season were more beneficial to tocopherol accumulation during almond lipid development. However, other studies reported that maize α-tocopherol had a large increase when average temperature rose 1°C in a greenhouse (Britz and Kremer 2002) and almonds grown in Spain had higher tocopherol concentration in the warm year of 2009 relative to the normal year of 2008 (Kodad et al. 2001).

In the wet season of 2010/11, results for the various tocopherol components suggested considerable but inconsistent treatment responses despite the lack of any apparent water stress and no obvious trend in line with the imposed deficits. In other species, water stress appears to promote tocopherol synthesis (Britz and Kremer 2002; Ali et al. 2009, 2010). The comparatively high value of tocopherol under 85% RDI is therefore especially puzzling because of a lack of water stress.
In 2011/12, little variation in almond tocopherols was observed between treatments except for the more severe deficit of 70% SDI which for all components except γ-tocopherol had higher values than the control (Table 4). However, there was no obvious relationship between almond tocopherols and the degree of water deficiency. This is in contrast to maize and sunflower whose tocopherol concentration was increased by drought (Ali et al. 2009, 2010). The minor tocol homologues appeared to be slightly more responsive to deficit irrigation than the main tocol homologue α-tocopherol, but their small proportion had little impact on the amount of total tocopherols.

3.4 Consideration of almond kernel size

Results after two seasons of field experimentation showed that moderate deficit irrigation (85% RDI and 85% SDI) had no negative impact on almond kernel size, individual kernel weights were: 1.450 g for control, 1.452 g for SDI 85%, 1.515 g for RDI 85%, and two way ANOVA analysis showed that there was no significant difference between those results. Meanwhile, there was no significant difference between control and other treatments like severe deficiency 70% and 55% ET₀, but there was a significant difference between RDI 85% (1.515 g), wet (1.505 g) and severe deficiency RDI 55% (1.410 g), RDI 70% (1.407 g) and SDI 55% (1.377 g). Therefore, compared to the control, the water deficit treatments had no effect on almond kernel size, under both moderate deficiency and severe deficiency. Comparably, kernel lipid content as an indicator of storage capability also remained unaffected by moderate water deficiency but was reduced by more severe deficiency particularly under regulated but not under sustained deficit strategy (Table 3).
CONCLUSIONS

In conclusion, unlike soybeans (Candogan et al. 2013), sunflower (Baldini et al. 2002; Ali et al. 2009), canola (Zarei et al. 2010) and peanuts (Dwivedi et al. 1996), almond kernel lipid quantity had no clear linear correlation with the amount of irrigation. The lack of a clear correlation relative to other annual crops may result from the fact that almond is a perennial tree crop with an extensive root system and therefore may be better buffered against short term water deficiency. Under moderate water deficiency, almond oil content was mostly stable, but decreased when trees were under severe water deficiency. Almond trees which received excess water (the wet treatment), did not produce big kernels and did not contain enhanced levels of either lipids or tocopherols. Excess water supply therefore was ineffective for kernel size and nutrient enhancement. Sustained deficit irrigation had little influence on tocopherol concentration, e.g. more water deficiency did not significantly increase or decrease Vitamin E concentration. Therefore, taking into consideration all the results of lipid and tocopherol concentration, as well as fatty acid composition, our study indicated that almond is not a water demanding plant and is capable of adapting to water deficient conditions. Almonds may be grown successfully using sustained deficit irrigation in Australia without detriment to their nutritional benefits. More work is needed in the future to confirm these findings.

ACKNOWLEDGEMENTS

We are grateful to David Apps and Dr. Robert Asenstorfer for technical support with GC and HPLC analysis. We also acknowledge the Department of Environment and Primary Industries Victoria, the Almond Board of Australia and Horticulture Australia Limited for the provision of funding.
REFERENCES


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Table 6. (Table 1. Timing of deficit irrigation treatments)

<table>
<thead>
<tr>
<th>strategy</th>
<th>Aug</th>
<th>Sept</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>gradient</th>
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<td>RDI</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>85%</td>
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<tr>
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<td></td>
<td>50% of control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50% of control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55%</td>
</tr>
<tr>
<td>SDI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at each irrigation 85%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>at each irrigation 70%</td>
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<td></td>
<td></td>
<td></td>
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<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at each irrigation 55%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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Table 7. (Table 2. Climatic conditions during the 2010/11 and 2011/12 growing seasons at the field trial located in Lake Powell (34.71°S, 142.93°E, elevation 53m))

<table>
<thead>
<tr>
<th>season</th>
<th>month</th>
<th>T.Max (°C)</th>
<th>T.Min (°C)</th>
<th>Rain (mm)</th>
<th>ETo (mm)</th>
<th>RHmaxT (%)</th>
<th>RHminT (%)</th>
</tr>
</thead>
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<tr>
<td>2010/2011</td>
<td>Aug</td>
<td>15.3</td>
<td>5.0</td>
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<td>6.0</td>
<td>29</td>
<td>59</td>
<td>46.2</td>
<td>95.6</td>
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<tr>
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<td>Sept</td>
<td>18.3</td>
<td>6.3</td>
<td>21</td>
<td>74</td>
<td>47.9</td>
<td>94.9</td>
</tr>
<tr>
<td>2011/2012</td>
<td>Sept</td>
<td>21.8</td>
<td>6.3</td>
<td>10</td>
<td>91</td>
<td>36.9</td>
<td>90.7</td>
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<tr>
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<td>9.6</td>
<td>70</td>
<td>114</td>
<td>41.9</td>
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</tr>
<tr>
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<td>24.8</td>
<td>10.5</td>
<td>35</td>
<td>112</td>
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<td>86.3</td>
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<td>33.8</td>
<td>83.4</td>
</tr>
<tr>
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<td>Dec</td>
<td>29.2</td>
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<td>34.2</td>
<td>79.9</td>
</tr>
<tr>
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<td>Dec</td>
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<td>15.3</td>
<td>32</td>
<td>204</td>
<td>29.1</td>
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</tr>
<tr>
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<td>79.9</td>
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<tr>
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<td>17.1</td>
<td>31</td>
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<td>30.0</td>
<td>70.3</td>
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<tr>
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<td>30.4</td>
<td>17.0</td>
<td>96</td>
<td>128</td>
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<td>86.1</td>
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<tr>
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<td>31.5</td>
<td>16.9</td>
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<td>32.9</td>
<td>75.6</td>
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<td>13.7</td>
<td>29</td>
<td>107</td>
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<td>2010/2011</td>
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<td>23.6</td>
<td>9.5</td>
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<td>42.7</td>
<td>96.7</td>
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<tr>
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<td>9.9</td>
<td>4</td>
<td>80</td>
<td>40.5</td>
<td>96.3</td>
</tr>
</tbody>
</table>
T.max and T.min are monthly averages calculated from daily max and min temp. RH.maxT and RH.minT are monthly averages calculated from daily RH at the max or min temp. Rain is cumulative monthly rainfall, ETo is cumulative monthly reference crop evapotranspiration.

Table 8. (Table 3. Effects of deficit irrigation on almond lipid content (g/100g) and fatty acid (percentage of total lipid))

<table>
<thead>
<tr>
<th>treatment</th>
<th>year</th>
<th>lipid content</th>
<th>myristic</th>
<th>palmitic</th>
<th>palmitoleic</th>
<th>stearic</th>
<th>oleic</th>
<th>vaccenic</th>
<th>linoleic</th>
<th>linolenic</th>
<th>arachidic</th>
<th>ratio o/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td></td>
<td>48.99 ± 2.93</td>
<td>0.05 ± 0.00</td>
<td>6.88 ± 0.01</td>
<td>0.46 ± 0.00</td>
<td>1.27 ± 0.00</td>
<td>61.89 ± 0.08</td>
<td>1.37 ± 0.00</td>
<td>26.09 ± 0.07</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.37 ± 0.01</td>
</tr>
<tr>
<td>wet</td>
<td></td>
<td>49.04 ± 1.47</td>
<td>0.05 ± 0.00</td>
<td>6.76 ± 0.01</td>
<td>0.46 ± 0.00</td>
<td>1.19 ± 0.00</td>
<td>62.41 ± 0.07</td>
<td>1.42 ± 0.00</td>
<td>25.77 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.42 ± 0.00</td>
</tr>
<tr>
<td>RD185%</td>
<td>2011</td>
<td>51.58 ± 2.52</td>
<td>0.04 ± 0.00</td>
<td>6.98 ± 0.00</td>
<td>0.50 ± 0.00</td>
<td>1.20 ± 0.00</td>
<td>61.85 ± 0.03</td>
<td>1.42 ± 0.00</td>
<td>25.96 ± 0.05</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.38 ± 0.01</td>
</tr>
<tr>
<td>RD170%</td>
<td></td>
<td>54.55 ± 0.35</td>
<td>0.04 ± 0.00</td>
<td>6.94 ± 0.02</td>
<td>0.49 ± 0.00</td>
<td>1.27 ± 0.00</td>
<td>62.07 ± 0.05</td>
<td>1.40 ± 0.00</td>
<td>25.76 ± 0.05</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.41 ± 0.01</td>
</tr>
<tr>
<td>RD155%</td>
<td></td>
<td>52.56 ± 1.70</td>
<td>0.04 ± 0.00</td>
<td>7.04 ± 0.07</td>
<td>0.49 ± 0.00</td>
<td>1.18 ± 0.01</td>
<td>61.86 ± 0.02</td>
<td>1.47 ± 0.01</td>
<td>25.92 ± 0.14</td>
<td>0.07 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>2.39 ± 0.01</td>
</tr>
<tr>
<td>SD185%</td>
<td></td>
<td>51.95 ± 2.05</td>
<td>0.04 ± 0.00</td>
<td>6.83 ± 0.01</td>
<td>0.49 ± 0.00</td>
<td>1.25 ± 0.00</td>
<td>62.80 ± 0.03</td>
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<td>25.21 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.49 ± 0.00</td>
</tr>
<tr>
<td>SD170%</td>
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<td>52.88 ± 2.80</td>
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<td>6.89 ± 0.01</td>
<td>0.50 ± 0.00</td>
<td>1.22 ± 0.01</td>
<td>62.11 ± 0.06</td>
<td>1.44 ± 0.02</td>
<td>25.85 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.01</td>
<td>2.40 ± 0.00</td>
</tr>
<tr>
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<td>52.34 ± 2.31</td>
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<td>6.91 ± 0.02</td>
<td>0.50 ± 0.00</td>
<td>1.21 ± 0.00</td>
<td>62.26 ± 0.10</td>
<td>1.45 ± 0.01</td>
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<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
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<tr>
<td><strong>NS</strong></td>
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<td>57.32 ± 0.82</td>
<td>0.04 ± 0.00</td>
<td>7.10 ± 0.00</td>
<td>0.46 ± 0.00</td>
<td>1.28 ± 0.01</td>
<td>62.77 ± 0.03</td>
<td>1.90 ± 0.00</td>
<td>25.20 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.49 ± 0.00</td>
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<tr>
<td><strong>NS</strong></td>
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<td>56.17 ± 0.08</td>
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<td>6.90 ± 0.00</td>
<td>0.47 ± 0.00</td>
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<td>62.75 ± 0.04</td>
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<td>24.70 ± 0.29</td>
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<td>2.54 ± 0.03</td>
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<tr>
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<td>2012</td>
<td>57.61 ± 1.71</td>
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<td>6.80 ± 0.00</td>
<td>0.44 ± 0.00</td>
<td>1.31 ± 0.01</td>
<td>64.30 ± 0.12</td>
<td>1.80 ± 0.00</td>
<td>24.03 ± 0.09</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
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<tr>
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<td>50.90 ± 1.57</td>
<td>0.05 ± 0.00</td>
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<td>0.47 ± 0.01</td>
<td>1.28 ± 0.00</td>
<td>59.53 ± 0.09</td>
<td>1.80 ± 0.00</td>
<td>28.20 ± 0.06</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
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<td>0.51 ± 0.03</td>
<td>1.41 ± 0.01</td>
<td>57.40 ± 0.21</td>
<td>1.83 ± 0.07</td>
<td>29.83 ± 0.33</td>
<td>0.06 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>1.92 ± 0.03</td>
</tr>
<tr>
<td>SD185%</td>
<td></td>
<td>55.40 ± 1.22</td>
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<td>7.13 ± 0.03</td>
<td>0.53 ± 0.02</td>
<td>1.35 ± 0.00</td>
<td>62.40 ± 0.06</td>
<td>1.93 ± 0.03</td>
<td>25.37 ± 0.03</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>2.46 ± 0.01</td>
</tr>
<tr>
<td>SD170%</td>
<td></td>
<td>52.29 ± 1.73</td>
<td>0.04 ± 0.00</td>
<td>7.30 ± 0.00</td>
<td>0.50 ± 0.00</td>
<td>1.23 ± 0.00</td>
<td>61.03 ± 0.07</td>
<td>2.00 ± 0.00</td>
<td>26.60 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.01</td>
<td>2.29 ± 0.00</td>
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<td>SD155%</td>
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<td>54.73 ± 1.23</td>
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<td>7.50 ± 0.10</td>
<td>0.49 ± 0.03</td>
<td>1.40 ± 0.02</td>
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<td>0.05 ± 0.00</td>
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<td>1.89 ± 0.03</td>
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</table>

**NS** *** * *** * *** NS ***
Mean value from triplicate performance ± standard error. *** p< 0.001, ** p<0.01, * p<0.05

Table 9. (Table 4. Effects of deficit irrigation on tocols)

<table>
<thead>
<tr>
<th>treatment</th>
<th>crop year</th>
<th>total tocols (mg/100g)</th>
<th>α-tocopherol (mg/100g)</th>
<th>γ-tocopherol (mg/100g)</th>
<th>α-tocotrienol (mg/100g)</th>
<th>β-tocopherol (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2011</td>
<td>21.07 ± 0.15</td>
<td>20.03 ± 0.98</td>
<td>0.57 ± 0.04</td>
<td>0.34 ± 0.02</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>wet</td>
<td>2011</td>
<td>20.71 ± 0.15</td>
<td>19.78 ± 0.15</td>
<td>0.50 ± 0.01</td>
<td>0.31 ± 0.00</td>
<td>0.13 ± 0.00</td>
</tr>
<tr>
<td>RDI 85%</td>
<td>2011</td>
<td>25.68 ± 1.68</td>
<td>24.80 ± 1.63</td>
<td>0.47 ± 0.02</td>
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<td>0.12 ± 0.01</td>
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<td>RDI 70%</td>
<td>2011</td>
<td>17.07 ± 0.60</td>
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<td>0.48 ± 0.02</td>
<td>0.30 ± 0.00</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td>RDI 55%</td>
<td>2011</td>
<td>16.82 ± 0.11</td>
<td>15.95 ± 0.10</td>
<td>0.46 ± 0.01</td>
<td>0.29 ± 0.00</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>SDI 85%</td>
<td>2011</td>
<td>17.88 ± 0.23</td>
<td>17.06 ± 0.21</td>
<td>0.52 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>SDI 70%</td>
<td>2011</td>
<td>20.23 ± 0.85</td>
<td>19.38 ± 0.82</td>
<td>0.48 ± 0.02</td>
<td>0.26 ± 0.03</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td>SDI 55%</td>
<td>2011</td>
<td>18.72 ± 1.10</td>
<td>17.87 ± 1.09</td>
<td>0.53 ± 0.02</td>
<td>0.25 ± 0.03</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Mean value from triplicate performance ± standard error. *** p< 0.001, ** p<0.01, * p<0.05
Figure 5. (Figure 1. Midday Stem Water Potential of Almond Trees)

Vertical bars indicate standard errors of the mean.

The stars above the vertical bars indicate statistical significance (5% level)
CHAPTER FOUR

Accumulation of Lipid and Tocopherols during Almond Kernel Development

Introduction

Horticulture based research often includes studies concerning the factors that influence fruit development, so as to understand the implications of management practices, such as pruning, irrigation, pest and disease management, canopy management, irrigation and fertilisation, on plant health and physiology, as well as crop yield, composition and quality. In the case of almond production, previous studies have included investigations into kernel development to determine almond lipid accumulation using techniques such as gas and liquid chromatography and proton isomer trace (Munshi et al. 1983, Soler et al. 1988). To date, these studies have only considered almonds grown in the northern hemisphere and not almonds grown under climate conditions of the southern hemisphere.

The following paper reports the accumulation of fatty acids and tocopherols during the development of almond kernels (cv. Nonpareil) grown in the Adelaide Plains region. Modern analytical technologies, i.e. gas chromatography and high performance liquid chromatography, were employed to monitor changes in almond composition with maturation, thereby improving the current understanding of fatty acid and tocopherol accumulation in almonds.

Ying Zhu, Kerry Wilkinson, Michelle Wirthensohn*  

University of Adelaide, School of Agriculture, Food and Wine, PMB 1, Glen Osmond, SA 5064

Email for Correspondence: michelle.wirthensohn@adelaide.edu.au

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ABSTRACT

This study investigated the changes in fatty acid and tocopherol levels during almond kernel lipid maturation, in the cultivar Nonpareil, grown in the Adelaide Plains of South Australia. It was the first time a study has been conducted on lipid development in almonds grown in the southern hemisphere. Six development stages were identified 74 days after anthesis, at 20 day intervals between each stage, from November 2012 to February 2013.

Almond lipid accumulation occurred more quickly in the first two periods than in the following three periods; with a maximum rate of 1.83 g/d per 100 g almond fresh kernels during the second period. Oleic acid synthesis followed a stable growth trend, with an average accumulation rate of 0.57% of total lipid content per day. However, linoleic acid accumulation showed a different trend: the concentration reached a maximum value in the second period and then declined the steady proportion of 23% of the final lipid content was reached. In addition, all tocopherols accumulated at a steady rate, and were positively correlated with lipid development. No interconversion points between tocopherol homologues were found.

The significance of this study is to identify the crucial timing of almond lipid development and tocopherol accumulation, in order to inform almond orchard management including irrigation, fertilization and other kernel quality improvement practices.

Keywords: almond, Prunus, fatty acid, tocopherol, kernel lipid accumulation
INTRODUCTION

Lipids are the major nutrient in almond kernels and account for more than 50% of the total kernel (dry weight). Almond lipids predominantly comprise the monounsaturated fatty acid, oleic acid; the polyunsaturated fatty acid, linoleic acid, and tocopherols. Collectively, these compounds play a lipophilic antioxidant role in in-vivo metabolism, as addressed in previous studies (Jambazian et al. 2005, Jenkins et al. 2006, Hollis and Mattes 2007, Rajaram et al. 2010, Wien et al. 2010, Damasceno et al. 2011). Given the significance of these compounds, it would be beneficial to understand their accumulation during almond kernel maturation and development. This would influence the timing of almond orchard management such as irrigation and fertilization to maximise kernel quality.

Previous studies on this topic include Soler et al. (1988) using gas liquid chromatography (GLC) technique reported the oil content and fatty acid composition of developing almond seeds and Munshi et al (1983) applying [1-14C] acetate to trace the biosynthesis of lipids in the developing almond kernels, as well as the latest study by Cherif et al. (2004) to investigate the difference of three cultivars on this topic. The study reported here aimed to investigate the kernel development of almonds grown in Australian inland climate condition, in order to assist orchard management in the southern growing regions, such as the technique to enhance almond kernel Vitamin E content and deficiency irrigation strategies for water saving measures in Australian almond orchards.
MATERIALS and METHODS

2.1 Plant Materials. 26-year-old almond trees (*Prunus dulcis*, cv. Nonpareil) grown in Angle Vale in the North Adelaide Plains (34°.92’S, 138°.60’E, elevation 48 m) were sampled. There was no special treatment for the selected trees, which were matintained according to the grower’s usual orchard management regime. The soil texture is red-brown earth in particular cracking clay. Almond fruits were harvested at six different stages starting 74 days after anthesis at 20 day intervals between each stage from November 2012 to February 2013. Sampling was based on 40 trees, two fruits each tree were randomly picked and 80 fruits in total were mixed together. The kernels were opened up for photographic imaging at each stage, and the fresh kernels were ground to a slurry. Only the fully ripened kernels from the sixth stage were tested in both fresh and dry condition. The drying conditions were 50°C for 48 hours and the moisture content (of approximately 2%) was measured by gravimetric technique. Dried kernels were ground to a fine powder, after sieving through a 1000 µM mesh and subjected to analysis.

2.2 Chemical Reagents. Hexane, ethanol, methanol, chloroform, *n*-heptane, sodium chloride, butylatedhydroxyanisole (BHA), sulphuric acid, ascorbic acid, potassium hydroxide were purchased from Merck, Scharlau and Sigma. C17 free fatty acid (C17 FFA from Nucheck Prep Inc.) was used as an internal standard to quantify the fatty acid percentage of almond lipids. For tocopherol identification and quantification, an α, β, γ, δ-tocopherol standard set (Calbiochem, Germany) and an α-tocotrienol standard (Cayman Chemicals, USA) were used to develop external standard curves.
2.3 Fatty Acids 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 reported by Makrides (1996) with some modification. Briefly, almond powder (0.05 g) was mixed with 0.9% aqueous sodium chloride (2 mL), methanol (3 mL containing 0.005% BHA), free fatty acid C17 (400 µL, 0.16% in methanol) as an internal standard and chloroform (6 mL), and then 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 drying, methylation was achieved by adding chloroform:methanol (9:1v/v, 1 mL; containing 0.005% BHA) and methanol (5 mL containing 1% sulphuric acid), and heating to 70°C for 3 hours. After the samples had cooled, n-heptane (2 mL), water (0.75 mL) was added and samples were mixed thoroughly. The organic layer was transferred to a GC vial. Fatty acid composition was determined using an HP 6890 Gas Chromatograph (Hewlett Packard, Palo Alto, CA. USA) equipped with a flame ionization detector (FID), HP 7683 autosampler, HP Chemstation software, split/splitless injection. A capillary GC column SGE BPX 70 (50 m, 0.32 mm ID, 0.25 µm) was used (SGE Analytical Science Pty. Ltd. Ringwood, VIC. Australia). Helium was the carrier gas and the split-ratio was 20:1, the injector temperature was set at 250°C and the detector temperature at 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 based on the retention time of the internal standard free fatty acid C17.
2.4 Tocol Determination. Tocol extraction was based on the alkaline saponification and hexane extraction method used previously for cereals and nuts (19). Briefly, almond powder (0.25 g) was mixed with ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% 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, vortexing periodically (every 10 min). The tubes were then placed in ice water for 5 min, Millipore water (1.5 mL) and hexane (2.5 mL) was added and vortexed for 30 s, then centrifuged (1000g at 20°C for 10 min). The hexane layer was transferred to vials and the residue extracted again, after extraction twice, the combined extract (in hexane) was evaporated using a nitrogen evaporator (N-EVAP 112) at 45°C, thereafter, hexane (1 mL) was added to each vial for HPLC analysis. The analysis protocol referred to Lampi and colleagues (2008, 2011), specifically, the isocratic mobile phase was hexane (with 2% 1,4-dioxane), flow rate 1.0 mL/min, injection volume 20 µL, column temperature 25°C. HPLC analysis was performed using an Agilent 1200 HPLC (Agilent Technologies, Deutschland, Germany) coupled with a diode array detector (DAD), fluorescence detector (FLD), autosampler, quaternary pump, Agilent Chemstation and Grace Alltime HP Silica column (150 mm, 3 mm, 3 µm) (Grace Discovery Sciences, Deerfield, IL, USA). α, β, γ, δ-Tocopherol and α-tocotrienol individual external standards were used for the calibration curves. α-Tocopherol was checked under DAD signal wavelength 292 nm while β, γ, δ-tocopherol and α-tocotrienol were checked under FLD signal wavelength 292 nm (excitation) and 325 nm (emission).

2.5 Data Analysis. Chemical data were analysed by ANOVA using GenStat (14th Edition, VSN International Limited, Herts UK) and GraphPad Prism 5 (Version 5.01 GraphPad Software, Inc. La Jolla, CA, USA). Mean comparisons were performed by least significant difference (LSD) multiple-comparison test at p < 0.05. Pearson’s co-efficient was used for correlation analysis.
RESULTS and DISCUSSION

3.1 Kernel and fruit appearance at different developmental stages

The appearance of almond kernels and fruit during the time course of almond fruit ripening, are shown in Figure 1. There are six stages to exhibit the physical appearances. At the first and second stage, almond kernels had a clear jelly interior with yellowish skin, and the outer hull was bright green in colour. As the kernel developed, the jelly interior solidified and became cream in colour by the third and fourth stages (156 days post anthesis), but the kernel skin was still pale and the fruit colour was unchanged. By the fifth stage, the kernel had become firm and the skin was brown. The fruit had become dry and exhibited a brown leathery texture. By the fully ripe stage (sixth) the fruit was completely dry. Similar anatomical observations were described by Munshi and Sukhja (1984), despite the fact that the characteristic of each stage in that study appeared 10 days earlier compared to this study, and the different varieties studied: i.e. Nonpareil for this study and a regional selection (h5) for their study.

3.2 Lipid accumulation at different development stages

The rate of lipid accumulation in 100 g fresh kernels is shown in Figure 2 and follows a sigmoidal pattern. Based on the rate differences, the pattern was divided into four developmental periods: period 1 from the first to the second stage (74 days to 95 days) having 0.38 g/100 g/day; period 2 from the second to the third stage (95 days to 115 days) having 1.83 g/100 g/day; period 3 from the third to the fifth stage (115 days to 156 days) having 0.05 g/100 g/day; period 4 from the fifth to the sixth stage (156 days to 167 days) having 0.62 g/100 g/day. During period 1 lipid
accumulation was slow; period 2 had the highest development of almond lipid synthesis, within 20 days, lipid synthesis increased to 46.46 g/100 g fresh kernels at stage 3, which is regarded as the critical time for lipid accumulation. Thereafter, the rate of almond lipid synthesis declined to 0.05g /100 g/day, where period 3 lasted 41 days. During this time, other components are being actively metabolised, too, for example, significant quantities of protein are formed, and sugar and moisture contents decrease, according to Soler’s et al. (1988) report. Approaching the ripening period, lipid accumulation increased again to 0.62 g/100 g/day and reached a final amount of 53.70 g/100 g dry kernels. This small rise could be partly due to the moisture decline and therefore the kernel dry mass was concentrated. The weather during this period had low rainfall and high temperatures (Table 1). Such climatic conditions can support the higher plants seed dry mass to accumulate (Monga et al. 1983, Munshi et al. 1983, Onemli 2012).

3.3 Fatty acid composition at different development stages

This study analysed the variations of almond free fatty acids during lipid accumulation, including myristic, palmitic, palmitoleic, vaccenic, stearic, oleic, linoleic, linolenic and arachidic (Table 2). Appreciably, the major fatty acids like oleic and linoleic in almond lipids were distinguished between the initial phase and the mature stage. Other minor fatty acids showed less difference during lipid development than the major fatty acids.

In addition, the major fatty acids, oleic and linoleic, had an inter-conversion point at the stage 2 (95 days post anthesis), specifically, both fatty acids’ concentration increased to 39% of total lipids from the first stage where their levels were 10.83% and 24.36% respectively. Thereafter, oleic acid was still synthesizing till it reached the maximum value at 63% of total lipids, and was
strongly correlated with lipid development (R=0.8651) (Table 3). On the other hand, linoleic acid concentration started to decline from that point, until it reached a stable level at 23% of total lipids when the kernel was fully ripened, and the concentration was not in association with lipid development (Table 3). In a study of fatty acid synthesis in sunflower seeds, Onemli (2012) reported a different situation: i.e. at the second development stage, the cross-point was the maximum value for oleic acid, rather than linoleic acid, thereafter, oleic acid concentration decreased. Yet, there is a similarity: no linear response of linoleic acid to sunflower oil accumulation was found but a negative correlation between oleic acid and sunflower oil content was observed. Moreover, in an early study of almond lipid development, oleic acid and linoleic acid exhibited the same trends as the present study during lipid accumulation (Soler et al. 1988). Noticeably, the concentration of linoleic acid was high at 59.2% of total lipids and then declined to 29% at maturity. Our study has not observed such high concentrations of linoleic acid during almond lipid maturation. A possible explanation could be that the first measurement in our study was later than in Soler’s study, which commenced at 23 days post anthesis, whereas we began to sample at 74 days post anthesis, thus, the highest concentration of linoleic acid may have already passed.

Another aspect of almond fatty acid synthesis is the pattern of linoleic acid accumulation during almond lipid maturation which was quite similar with the saturated fatty acids in this study, such as myristic, palmitic and arachidic, e.g. the concentration reached the maximum value at the very early stage and then decreased till it reached a stable value when the lipids were fully formed. A similar finding was reported by Munshi and Sukhija (1984) who applied $^{14}$C to track almond lipid biosynthesis. The correlation coefficient between fatty acids and lipid content, as well as between individual fatty acids is shown in Table 3. A strong positive correlation was found between
vaccenic and palmitic (R=0.9592), followed by the correlation between oleic and palmitic (R=0.8828). It could be supposed that there exists some metabolic pathway between C18:1 and C16:0 in almond lipid maturation. It has not been reported in the literature.

3.4 Tocopherol profile at different development stages

The relative composition of tocopherols has been well documented in fully ripened almond kernels (Kornsteiner et al. 2006, Lopez-Ortiz et al. 2008, Matthaus and Ozcan 2009, Kodad et al 2011, Madawala et al. 2012), however, such information was very limited in immature almond kernels. In other words, the synthesis trend of tocopherol homologues during almond lipid development was not well known. This study investigated the changes in four tocopherol homologues at six stages of almond kernel development, in order to analyse the key time of tocopherol accumulation during lipid synthesis. Figure 3 shows the linear regression between the tocopherol and almond lipid accumulation. Specifically, α-tocopherol (which represents total tocopherols) concentration showed a very strong positive correlation with almond lipid accumulation (R=0.864, p<0.0001). β-Tocopherol and α-tocotrienol also showed a strong positive correlation with lipid accumulation (R=0.824, 0.761 respectively, p<0.0001), while γ-tocopherol showed a moderate correlation with almond lipid accumulation (R=0.502 p = 0.02).

Through the whole kernel development, the transformation between the homologues was not observed. For example, from the early stage to the final stage, α-tocopherol was always the predominant constituent, no other homologues such as γ-tocopherol and α-tocotrienol were higher than α-tocopherol. In other words, it was not supposed that α-tocopherol was synthesized via γ-tocopherol methyltransferase or α-tocotrienol reduction. Therefore, it remained a question
to explore the metabolism pathway for almond tocopherols. A simple solution could be that a further investigation should commence at an earlier stage, in order to search out the possible interconversion point.

In the present study, the trend of $\alpha$-tocopherol synthesis was divided into three periods: period 1 from the first stage to the second stage (74 days to 95 days after anthesis) 0.58 mg/day in 100g lipids; period 2 from the second stage to the fourth stage (from 95 days to 135 days post anthesis) 0.09 mg/day in 100g lipids and period 3 from the fourth stage to the final stage (from 135 days to 167 days post anthesis) 0.28 mg/day in 100g lipids. The highest accumulation rate took place in period 1. We see differences between the rate of $\alpha$-tocopherol synthesis and lipid accumulation: the former occurred from the first stage to the second stage and the latter happened from the second stage to the third stage, despite there being a positive correlation between $\alpha$-tocopherol synthesis and lipid accumulation ($R=0.864$, $p<0.0001$). Future research could involve compositional analysis of samples harvested at earlier stages of almond kernel development.

**CONCLUSIONS**

This study was an exploratory study on kernel lipid development, changes in fatty acid composition and relative composition of tocopherols during lipid accumulation for almonds grown in the Adelaide Plains in Australia. We have shown that the key periods for almond lipid accumulation and tocopherol synthesis are between 95 and 115 days, and 74 and 95 days post anthesis, respectively. The time period from 74 to 115 days post anthesis is a crucial period to apply orchard management techniques to enhance the major nutrients in almond, the lipids and tocopherols.
ACKNOWLEDGEMENTS

We thank the local grower Vincent Ruggerio for supporting the field work. We are grateful to David Apps and Dr. Robert Asenstorfer with help on the GC and HPLC analysis.

REFERENCES


First stage

Second stage

Third stage

Figure 6. (Figure 1. Kernel appearance at different development stages)
Fourth stage

Fifth stage

Sixth stage

Figure 7. (Figure 1. Kernel appearance at different development stages) cont.
Figure 8. (Figure 2. Lipid accumulation at different stages of kernel development)

Figure 9. (Figure 3. Linear regression between tocol homologues and lipid accumulation)
Table 10. (Table 1. Climatic condition during 2012-2013 growing season at Adelaide Plains (34.92°S, 138.60°E, elevation 48 m))

<table>
<thead>
<tr>
<th>Year</th>
<th>Month</th>
<th>Maximum T (°C)</th>
<th>Minimum T (°C)</th>
<th>Rainfall (mm)</th>
<th>Solar radiation (MJm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>19.1</td>
<td>9.0</td>
<td>21.6</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>21.9</td>
<td>9.6</td>
<td>15.6</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>26.6</td>
<td>14.5</td>
<td>16.4</td>
<td>28.9</td>
</tr>
<tr>
<td></td>
<td>December</td>
<td>27.0</td>
<td>15.5</td>
<td>13.6</td>
<td>30.3</td>
</tr>
<tr>
<td>2013</td>
<td>January</td>
<td>28.5</td>
<td>15.7</td>
<td>9.0</td>
<td>27.6</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>28.7</td>
<td>17.3</td>
<td>12.4</td>
<td>23.7</td>
</tr>
</tbody>
</table>

Data from the website www.bom.org.au
### Table 11. Variations in fatty acid compositions at different stages of almond kernel development

<table>
<thead>
<tr>
<th></th>
<th>15/11/2012</th>
<th>5/12/2012</th>
<th>25/12/2012</th>
<th>15/01/2013</th>
<th>4/02/2013</th>
<th>15/02/2013</th>
<th>15/02/2013 (dry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>74 days</td>
<td>95 days</td>
<td>115 days</td>
<td>136 days</td>
<td>156 days</td>
<td>167 days</td>
<td>167 days (dry)</td>
</tr>
<tr>
<td>Myristic (C14:0)</td>
<td>0.00 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>7.65 ± 0.11</td>
<td>8.97 ± 0.05</td>
<td>8.14 ± 0.09</td>
<td>7.33 ± 0.02</td>
<td>7.20 ± 0.03</td>
<td>7.33 ± 0.05</td>
<td>7.31 ± 0.04</td>
</tr>
<tr>
<td>Palmitoleic (C16:1)</td>
<td>0.00 ± 0.00</td>
<td>0.40 ± 0.00</td>
<td>0.53 ± 0.01</td>
<td>0.48 ± 0.00</td>
<td>0.51 ± 0.01</td>
<td>0.50 ± 0.00</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>Data Missing</td>
<td>0.98 ± 0.02</td>
<td>1.42 ± 0.02</td>
<td>1.80 ± 0.02</td>
<td>1.74 ± 0.01</td>
<td>1.65 ± 0.03</td>
<td>1.63 ± 0.01</td>
</tr>
<tr>
<td>Vaccenic (C18:1n=7)</td>
<td>0.75 ± 0.07</td>
<td>1.36 ± 0.00</td>
<td>1.49 ± 0.01</td>
<td>1.38 ± 0.01</td>
<td>1.40 ± 0.00</td>
<td>1.43 ± 0.01</td>
<td>1.39 ± 0.01</td>
</tr>
<tr>
<td>Oleic (C18:1n=9)</td>
<td>10.83 ± 0.71</td>
<td>39.13 ± 0.17</td>
<td>52.44 ± 0.30</td>
<td>60.13 ± 0.24</td>
<td>63.31 ± 0.09</td>
<td>63.68 ± 0.18</td>
<td>62.55 ± 0.16</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>24.36 ± 1.62</td>
<td>38.49 ± 0.23</td>
<td>33.70 ± 0.15</td>
<td>26.78 ± 0.28</td>
<td>23.87 ± 0.06</td>
<td>23.42 ± 0.16</td>
<td>24.73 ± 0.15</td>
</tr>
<tr>
<td>Linolenic (C18:3)</td>
<td>0.00 ± 0.00</td>
<td>0.32 ± 0.02</td>
<td>0.11 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Arachidic (C20:0)</td>
<td>0.00 ± 0.00</td>
<td>0.11 ± 0.00</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.00</td>
</tr>
</tbody>
</table>

Values are means of three replicates ± standard error. Fatty acid content as a percentage of lipids.
### Table 12. (Table 3. Correlation coefficients between individual fatty acid and lipid as well as between each individual fatty acid)

<table>
<thead>
<tr>
<th>lipids</th>
<th>myristic (C14:0)</th>
<th>palmitic (C16:0)</th>
<th>palmitoleic (C16:1n-7)</th>
<th>vaccenic (C18:1n-7)</th>
<th>oleic (C18:1n-9)</th>
<th>linoleic (C18:2n-6)</th>
<th>arachidic (C20:0)</th>
<th>linolenic (C18:3 n-3)</th>
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</thead>
<tbody>
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<td>lipids</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>myristic</td>
<td>0.2016*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>palmitic</td>
<td>0.3624**</td>
<td>0.148NS</td>
<td>1</td>
<td></td>
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<td></td>
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<tr>
<td>palmitoleic</td>
<td>0.6680***</td>
<td>0.7342***</td>
<td>0.0101NS</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>vaccenic</td>
<td>0.5513***</td>
<td>0.8006***</td>
<td>0.0012NS</td>
<td>0.9592***</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oleic</td>
<td>0.8651***</td>
<td>0.4145**</td>
<td>0.1678 NS</td>
<td>0.8828***</td>
<td>0.7829***</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>linoleic</td>
<td>0.1489</td>
<td></td>
<td></td>
<td></td>
<td>0.3696**</td>
<td>0.8734***</td>
<td>0.01985 NS</td>
<td>0.07582NS 0.0329 NS</td>
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<tr>
<td>arachidic</td>
<td>0.3175**</td>
<td>0.7751***</td>
<td>0.0401NS</td>
<td>0.8014***</td>
<td>0.8454***</td>
<td>0.6042***</td>
<td>0.142NS</td>
<td>1</td>
</tr>
<tr>
<td>linolenic</td>
<td>0.0898</td>
<td></td>
<td></td>
<td></td>
<td>0.3811**</td>
<td>0.731***</td>
<td>0.06515 NS</td>
<td>0.1295NS 0.0000 NS 0.7332*** 0.3255**</td>
</tr>
</tbody>
</table>

Pearson r value presents to indicate significant correlations (CI 95%, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001)
CHAPTER FIVE

Influence of Solar UV Radiation on Fatty Acid and Tocopherol Concentration of Almond

Introduction

Australia is well known for its high levels of solar radiation. The measured ambient solar ultraviolet radiation (solar UVR) in Australia is 12-15% higher than for similar latitudes in the northern hemisphere (Gies et al. 2004). There was a common misconception that Australia’s strong UV radiation is caused by ozone depletion. However, the true reason is the geographic location and meteorological conditions (Roy et al. 1995, Herman et al. 1998, Gies et al. 2003, 2004).

The Australian continent has a high UVR index, because the elliptical orbit between the Earth and Sun makes the southern hemisphere location closer to the sun by 1.7% than the average sun-earth distance in January, which results in a 7% difference in solar UVR intensity for the Australian summer (Roy et al. 1995, Herman et al. 1998, Gies et al. 2003, 2004). In addition, clear-sky meteorological conditions make Australia more exposed to the sun, without the cloud cover, which can reduce UV radiation by 5% in the northern hemisphere (Herman et al. 1998, Kerr et al. 2002, Gies et al. 2004). In other words, the strong UV radiation will not change despite the ozone layer improving.
Excessive exposure to UVR is harmful for humans, however, research has found that moderate doses of UV radiation can stimulate the protective mechanisms in plants (Kakani et al. 2003), i.e. physical changes include leaf thickness, epidermal wax and hairiness (Steinmuller & Tevini 1985; Bornman & Vogelman 1991; Nagel et al. 1998; Manetas 2003), and changes in plant cell metabolism pathways and chemical substances (Olsson et al. 1998, Alexieva et al. 2001, Costa et al. 2002, Martz et al. 2007, Albert et al. 2010, Josuttis et al. 2010).

The following paper describes an investigation into the influence of solar radiation on almond composition and reports improved tocopherol levels of almonds harvested from trees that were exposed to increased levels of UV radiation.
Paper: Effect of Solar UV Radiation on Almond Tocopherols and Fatty Acids

Ying Zhu¹, Kerry Wilkinson¹, Kerryn King², John Javorniczky² and Michelle Wirthensohn¹*

¹ The University of Adelaide, School of Agriculture, Food and Wine, PMB 1 Glen Osmond, SA 5064, Australia

² Non-ionising Radiation Section, Radiation Health Services Branch, Australian Radiation Protection and Nuclear Safety Agency, 619 Lower Plenty Road Yallambie, VIC 3085, Australia

* Email for Correspondence: michelle.wirthensohn@adelaide.edu.au

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ABSTRACT

This study investigated the effects of solar UV radiation on almond (*Prunus dulcis*, cv. Nonpareil) lipid and tocopherol (Vitamin E) content, as well as fatty acid composition. Increased solar UV radiation was achieved by placing white weed mat on the ground in an almond orchard. UV radiation measurements indicated a solar radiation increase of 14% was achieved in the canopy. Under those conditions, almond α-tocopherol concentration was increased by 30%, but other tocol homologues such as γ-tocopherol, β-tocopherol and α-tocotrienol were not significantly changed by the same exposure. Almond kernel lipid content was not changed, but fatty acid composition was slightly altered: linoleic acid concentration decreased significantly by 2% and oleic acid increased by 0.8%, myristic acid increased by 10.7%, but other fatty acids were not changed. This study also examined the influence of position of the kernel within the tree canopy on the concentration of those compounds. Canopy position had no impact on α-tocopherol, γ-tocopherol and α-tocotrienol concentrations, but β-tocopherol was slightly higher in kernels located within the inner canopy. Lipid concentration of almonds harvested from the inner and outer canopy was not different, while linoleic acid was slightly lower in almonds within the inner canopy by 2% and oleic acid higher in the inner canopy by 1.5%, myristic acid was much lower in the inner canopy by 9.5%. The outcomes of this study suggested that enhancing solar UV radiation in almond orchards can increase almond Vitamin E content, and this practice therefore offers a cost-effective technique for improving almond kernel quality.

**KEYWORDS:** almond, *Prunus dulcis*, tocopherol, Vitamin E, solar UV radiation
INTRODUCTION

Australia is located in the southern hemisphere, and such geographic conditions plus clear skies result in Australia having 12-15% higher solar UV (ultra-visible) radiation than similar latitudes in the northern hemisphere (Roy et al. 1995, Herman et al. 1998, Gies et al. 2004). Research has found UV radiation can change a plant’s morphological structure and physiological metabolism (Kakani et al. 2003). According to previous studies, moderate doses of UV radiation can stimulate protective mechanisms in plants such as increased leaf thickness, epidermal wax and hairiness (Olsson et al. 1999, Manetas 2003, Martz et al. 2007); and increased concentrations of antioxidant compounds such as flavonoids and anthocyanins. (Tevini et al. 1981, Olsson et al. 1999, Josuttis et al. 2010, Berli et al. 2011). The studies supplemented UV exposure using UV lamps to increase UV radiation (Han et al. 2004, Tevini et al. 1981, Olsson et al. 1999, Gao and Zhang 2008), or reduced UV exposure using UV blocking films (Baumbusch et al. 1998, Kolb et al. 2001, Xu et al 2008, Martz et al. 2007, Albert et al. 2010, Josuttis et al. 2010, Hakala-Yatkin et al. 2010). Very few studies utilized natural solar UV radiation to increase the amount of UV radiation and examine its effects on plant physiology. Green and colleagues (1995) applied aluminium foil to reflect light up the canopy of into apple trees and measured PAR (photosynthetically active radiation) and photosynthesis, and reported that the increased photosynthesis was positively related to increases in PAR. Pliakoni and colleagues (2010) adopted reflective mulch to investigate the influence of UV radiation levels on the phenolic acid composition of nectarines.

Given the importance of tocopherols and fatty acids to the nutritional value of almonds, this study investigated the influence of increased solar UV radiation on almond tocopherol and fatty acid
composition. It is well-known that almonds provide a rich source of Vitamin E (tocopherols) which is beneficial to the health of regular almond consumers (Spiller et. al. 1998, Jenkins et al. 2008, Rajaram et al. 2010, Damasceno et al. 2011), and also plays a role in preventing almond kernel rancidity due to free radicals (Socias i Company et al. 2010). Thus, it would be useful for research and industry alike, to investigate the influence of solar UVR on almond tocopherols, given their significance.

This study also examined almond lipid content and fatty acid composition affected by solar UV radiation, that largely feature in the almond nutrition portfolio. Almond lipids account for more than 50% of total kernel weight, and is comprised of the monounsaturated fatty acid, oleic acid (approximately 60-70%) and polyunsaturated fatty acid linoleic acid (20-30%), as well as other saturated fatty acids including stearic acid, palmitic acid arachidic acid and unsaturated fatty acids including myristic acid, palmitoleic acid which make up less than 10% of almond lipid fatty acids (Sathe et al. 2008, Matthaus and Ozcan 2009, Piscopo et al. 2010, Moayedi et al. 2010, Kodad et al. 2010, Yada et al. 2011). The high proportion of unsaturated fatty acid in almond lipid has also been demonstrated to perform medicinal functions for regular almond consumers, such as lowering cholesterol to improve blood kinetic condition (Hyson et al. 2002, Hollis et al. 2007, Wien et al. 2010). Similar to tocopherols, it would be advantageous to investigate the influence of solar UV radiation on almond fatty acid composition.

MATERIALS AND METHODS

2.1 Plant Material. 15-year-old Nonpareil almond trees were investigated in Adelaide, South Australia (34.97°S, 138.64°E, elevation 100 m) during the 2012/13 growing season. The soil type
is red brown earth with 25 cm of fine sandy loam topsoil and prismatic structure clay subsoil (Litchfield 1952). Four trees were used for the experiment: two trees growing in normal conditions (control treatment) and two trees with white weed mats under the entire canopy to increase the total Solar UV radiation exposure to the trees by reflection into the understory from anthesis till harvest.

2.3 UV radiation measurement. UVR was measured using polysulphone UV badges as described in previous studies (Parisi and Wong 2000, Heisler et al. 2003, Webb 2003, Parasi et al. 2010). The UV badges used in this study were provided by the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA), with a 7 mm exposure aperture in a light cardboard frame. Polysulphone film badges were measured pre- and post- UV exposure at 330 nm in a Cary 50 UV-Vis spectrophotometer (Varian, Australia). The UV badges were placed in four positions: 1.5 m above the grass measuring full solar UV radiation; the north side outer canopy of the control tree; the north side inner canopy of the control tree. In these three positions, each UV badge was tied to the branches and a pole, parallel with the ground and facing the sunlight. The fourth position was in the middle canopy of a reflected tree, placed beneath thick cardboard, with the film facing the white weed mat to measure the extra amount of reflected solar UV radiation. Measurements were carried out from Oct. 2012 (anthesis) to Feb. 2013 (harvest) three times per month for 1 hour duration at each trial on a clear sky day. The total exposure of each badge was determined and compared to a dose response curve determined previously by ARPANSA.
2.4 **Sampling condition.** Almonds were harvested from the inner and outer canopies of each tree from all four quadrants of the compass. The inner canopy kernels were picked from inner branches and were within 30 cm of the trunk; outer canopy kernels were picked from the outer 30 cm end of outer branches. Fruits were sampled from each tree, 30 from both inner canopy and outer canopies. Samples were categorised as four groups: control inner, control outer, reflect inner and reflect outer. The samples in each group were run in triplicate.

2.4 **Chemical Reagents.** Hexane, ethanol, methanol, chloroform, n-heptane, sodium chloride, butylatedhydroxyanisole (BHA), sulphuric acid, ascorbic acid, potassium hydroxide were purchased from Merck, Scharlau and Sigma. C17 free fatty acid (C17 FFA from Nucheck Prep Inc.) was used as an internal standard to quantify the fatty acid percentage of almond lipids. For tocopherol identification and quantification, an α, β, γ, δ-tocopherol standard set (Calbiochem, Germany) and an α-tocotrienol standard (Cayman Chemicals, USA) were used to develop external standard curves.

2.5 **Sample Preparation.** Kernels were dried at 50°C for 48 hours, ground to a fine powder and sieved through a 1000 μm mesh, then stored under nitrogen in glass vials until required for analysis.

2.6 **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 reported by Makrides (18) with some modification. Briefly, almond powder (0.05 g) was mixed with 0.9% aqueous sodium chloride (2 mL), methanol (3 mL containing 0.005% BHA), free fatty acid C17 (400 μL, 0.16% in methanol)
as an internal standard and chloroform (6 mL), and then 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 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 hours. After the 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. Fatty acid composition was determined using a HP 6890 Gas Chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID), HP 7683 autosampler, HP Chemstation software, split/splitless injection. A capillary GC column SGE BPX 70 (50 m, 0.32 mm ID, 0.25 μm) was used (SGE Analytical Science Pty. Ltd. Ringwood, VIC, Australia). Helium was the carrier gas and the split-ratio was 20:1, the injector temperature was set at 250°C and the detector temperature at 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 based on the retention time of the internal standard free fatty acid C17.

2.7 Tocol Determination. Tocol extraction was based on the alkaline saponification and hexane extraction method used previously for cereals and nuts (Xu 2002). Briefly, almond powder (0.25 g) was mixed with ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% 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, vortexing periodically (every 10 min). The tubes were then placed in ice water for 5 min, water (1.5 mL) and hexane (2.5 mL) were added and vortexed for 30 s, then centrifuged (1000 g at 20°C for 10 min). The hexane layer was transferred to vials and the residue extracted again. The hexane was evaporated from the combined extractions using a
nitrogen evaporator (N-EVAP 112) at 45°C, and hexane (1 mL) was added to each vial for HPLC analysis. The analysis protocol was based on methods reported by Lampi and colleagues (2008, 2011), specifically, the isocratic mobile phase was hexane (with 2% 1,4-dioxane), flow rate 1.0 mL/min, injection volume 20 μL, column temperature 25°C. HPLC analysis was performed using an Agilent 1200 HPLC (Agilent Technologies, Deutschland, Germany) coupled with a diode array detector (DAD), fluorescence detector (FLD), autosampler, quaternary pump, Agilent Chemstation and Grace Alltime HP Silica column (150 mm, 3 mm, 3 μm) (Grace Discovery Sciences, Deerfield, IL. USA). α, β, γ, δ-Tocopherol and α-tocotrienol individual external standards were used for preparation of calibration curves. α-Tocopherol was analysed under DAD signal wavelength 292 nm while β, γ, δ-tocopherol and α-tocotrienol were analysed under FLD signal wavelength 292 nm (excitation) and 325 nm (emission).

2.8 Data Analysis. Chemical data were analysed by ANOVA using GenStat (14th Edition, VSN International Limited, Herts UK). Mean comparisons were performed by least significant difference (LSD) multiple-comparison test at p < 0.05.

RESULTS AND DISCUSSION

3.1 Solar UV radiation measurement

Solar UV radiation can be easily reflected by white surfaces, as demonstrated in previous studies, including grasslands 0.8%-1.6%; soil, clay 4.0%-6.0%; white house paint 22.0%; sea surf, white foam 25.0%-30.0%; snow 50.0%-88.0% (Sliney 1986). Adelaide Hills apple growers increase the colour intensity of their fruits using white weed mat to reflect solar UV radiation. Based on these
facts, we designed the field trial by placing white weed mat under two almond trees, cv. Nonpareil, and placing UV badges on the tree branches and on a pole above the grass near the trees for 1 hour during solar noon on days with a clear sky. The results of the UV radiation measurements are shown in Table 1. On average, full sun solar UV radiation was 16.9 SEDs (Standard Erythema Dose 100 Joule/m^2 is a measurement unit of UV radiation.) With white weed mat reflection, solar UV radiation around the outer canopy increased by 6.5% and within the inner canopy by 44.4%. On average, solar UV radiation was increased by 14% within the trees with weed mat beneath. Therefore, the trees surrounded by white weed mat absorbed more UVR than the control trees surrounded by grass. Solar UV radiation increment ranged from outer canopy to inner canopy because of branch shadowing and the sun movement during the day. The result is comparable with the solar UV radiation reflection by white house paint 22% (Sliney 1986). These results demonstrated that white weed mat can be used to increase solar UV radiation in the field.

3.2 Effect of increased solar UV radiation on almond tocopherols

Tables 2 and 4 display the solar UV radiation effect on almond tocopherols including α, β, δ-tocopherols, α-tocotrienol, and the influence of tree canopy position on those compounds. Specifically, ANOVA showed that α-tocopherol concentration in almond Nonpareil increased up to 30% in the trees surrounded by the white weed mat which received 14% extra solar UV radiation than the control. However, β, δ-tocopherols and α-tocotrienol concentrations were not affected by the extra solar UV radiation. The large increase of α-tocopherol concentration led to an equivalent rise in total tocopherol concentration because of the predominant proportion of α-tocopherol in almond total tocopherols. As for the canopy influence, the results showed no
difference on α-tocopherol, δ-tocopherol and α-tocotrienol, while there was a slight difference in β-tocopherol (inner higher than outer $p< 0.05$).

A possible explanation for the increase of α-tocopherol concentration is the mild solar UV radiation supplement (14%) using the white weed mat, which may have altered metabolic pathways and stimulated α-tocopherol accumulation in almond lipids. According to Josuttis’ study (2010), solar UV radiation enhanced strawberries flavonoids in the open-field compared to solar UV radiation blocked strawberries. Flavonoids increased under open-field condition were also reported in grapevine leaves, berries and soybean, by comparison with UV-exclusion conditions (Kolb et al. 2001, Xu et al. 2008, Berli et al. 2011). In addition, Pliakoni’s study (2010) reported that reflected sun light significantly increased total phenolics in nectarines. These findings support the notion that a mild increase in solar UV radiation could be a positive abiotic stress to trigger the plants’ defence system. Previous studies showed that flavonoid absorption occurs between 270-290 nm, which lies between the wavelengths of UVC (200-280 nm) to UVB (280-315 nm) whereby, such compounds could accumulate in the treated plants more than in the control plants and act as UV-protectors (Xu et al. 2008, Berli et al. 2011). In fact, UVC hardly reaches the Earth’s surface because stratospheric ozone completely absorbs UVC (Frederick et al. 1994), then compounds with absorption wavelengths in the UVB range indeed have a protective function. Noticeably, tocopherols absorption wavelength is 292 nm, which fits within the range of UVB wavelength (280-315 nm), therefore, it is reasonable to conclude that a moderate dose of solar UV radiation stimulated almond tocopherols accumulation to perform a protective role for the fruit. However, in contrast, a high dose UV radiation supplementation by UV lamps easily caused plants’ physiological and structural damages (Costa et al. 2002, Gao and Zhang 2008).
where the doses used were 300 SEDs and 0.04W/m² (equal to 35 SEDs), respectively, which were much more than our trees received in solar UV radiation (16 SEDs).

3.3 Effect of increased solar UV radiation on almond lipid content and fatty acid composition

Nonpareil almond lipid content was not altered by the increased solar UV radiation, (Tables 3 and 5). However, the increased UVR affected almond lipid fatty acid composition. Unsaturated fatty acids were more susceptible to solar UVR than saturated fatty acids. Levels of saturated fatty acids such as palmitic acid, stearic acid and arachidic acid were not affected by the increased solar UVR, but USFA showed more variability: myristic acid, oleic acid and linoleic acid were influenced by the extra solar UVR. Myristic acid was significantly increased by 10.7% (p<0.005), while linoleic acid and oleic acid were significantly decreased by 2% (p<0.001) and increased by 0.8% (p<0.05), respectively, (Tables 3 and 5).

Regarding canopy influence on lipid content and fatty acid composition, there was no difference in lipid content of almond harvested from the inner or outer canopy. However, most fatty acids showed differences between the inner and outer canopy. Myristic acid concentration increased significantly in kernels harvested from the outer canopy compared to those from the inner canopy by 9.5% (Tables 3 & 5). This is similar to the increase in kernels which received higher UVR due to the reflective weed mat. Linoleic acid concentration was significantly lower by 2.8% on inner canopy than the outer canopy, which is in contrast with the decrease in concentration with higher solar UV radiation, likewise, oleic acid concentration in inner canopy kernels was significantly higher by 1.5% than outer canopy kernels, which is also in contrast with the increase in oleic acid concentration with higher solar UV radiation. The contrasting results may be due to different
irradiance wavelengths being filtered through the leaves to the fruits and so the wavelengths involved in fatty acid metabolism may differ (Kolb et al. 2001, Xu et al. 2008) thus giving a canopy and solar UV radiation interaction. For example, stearic acid and vaccenic acid were not affected by different solar UV radiation levels, but both fatty acids showed different concentrations between inner canopy and outer canopy, (Table 5). Palmitic acid, oleic acid and linoleic acid showed to be influenced by canopy and solar UV radiation interaction, (Table 5). In comparison with the 30% increase in almond tocopherols, the slight change in fatty acid composition would not have large impacts on almond lipid nutrition.

It is necessary to mention that we did not investigate the kernel yield difference between treatments, nor did we measure PAR (photosynthesis activity radiation), as we aimed to investigate the effect of extra solar UV radiation on almond major nutrients. Some studies in the past stated that high UV radiation may reduce crop yield (Fedina and Velitchkova 2009). At the time they were concerned with ozone depletion and the subsequent higher UV radiation expected. However, others also reported vegetation biomass was increased under natural UV radiation compared with a UVB block out filter treatment (Cybulski III and Peterjohn 1999, Papadopoulos et al. 1999). In fact, those studies found that herbaceous and annual plants were susceptible to UV radiation while perennial crops gained more biomass under natural UV radiation compared to UVB exclusion conditions. In other words, a reasonable increased dose of UV radiation stimulated perennial plants defence mechanisms and increased antioxidant levels as well as biomass yield (Xu et al. 2008). In addition, according to Green’s study (1995) they found that aluminium foil ground covering increased PAR by 40% resulting in up to 34% increased rates of photosynthesis in the canopy. Reasonably, we can assume that almond kernel yield could be increased by a corresponding increase in solar UV radiation.
CONCLUSIONS

In conclusion, based on the field trial, the enhanced tocopherol concentrations (30%) and minimum alterations in fatty acid composition achieved by reflecting solar UV radiation into the tree canopy, could enable almond orchard management to improve almond kernel quality and therefore produce high profile nutritional almonds.

ACKNOWLEDGEMENTS

We are grateful to David Apps and Dr. Robert Asenstorfer for help with GC and HPLC analysis. We also give thanks to Dr. Ronald Smernik for providing constructive advice on preparation of this article.

REFERENCES


Table 13. (Table 1: UV Radiation data in Waite Almond Cage (measurement unit is SEDs: Standard Erythema Dose 100 Joule/m$^2$))

<table>
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<tr>
<th>Date</th>
<th>Time on</th>
<th>Time off</th>
<th>Reflect</th>
<th>Full Sun</th>
<th>Control Outer Canopy</th>
<th>Control Inner Canopy</th>
<th>Reflect Outer Canopy</th>
<th>Reflect Inner Canopy</th>
<th>UVR Increase Outer Canopy (%)</th>
<th>UVR Increase Inner Canopy (%)</th>
<th>UVR Increase Average (%)</th>
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<td>1.8</td>
<td>14.9</td>
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<td>38.6</td>
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<td>14:10</td>
<td>1.0</td>
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<td>15.8</td>
<td>1.3</td>
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<td>3.0</td>
<td>6.5</td>
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Note: R = C_o + F, R = C_i + F, Extr_o = R/C_o, Extr_i = R/C_i, Extr = (Extr_o + Extr_i) * 100 / 360.
<table>
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<th>α-tocopherol</th>
<th>γ-tocopherol</th>
<th>β-tocopherol</th>
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<td>outer</td>
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<tr>
<td></td>
<td>14.5 ± 1.47</td>
<td>14.8 ± 1.70</td>
<td>0.5 ± 0.04</td>
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The unit of concentration is mg/100g.
Values are means of triplicate performance ± standard errors.
Table 15. (Table 3. Fatty acid composition in each treatment)

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<th>UVR</th>
<th>lipid content</th>
<th>myristic acid</th>
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<tr>
<td>control</td>
<td>50.7 ± 2.02</td>
<td>52.8 ± 1.71</td>
<td>0.039 ± 0.00</td>
<td>0.043 ± 0.00</td>
<td>7.14 ± 0.14</td>
</tr>
<tr>
<td>reflect</td>
<td>51.1 ± 2.94</td>
<td>50.7 ± 1.72</td>
<td>0.044 ± 0.00</td>
<td>0.047 ± 0.00</td>
<td>6.89 ± 0.01</td>
</tr>
</tbody>
</table>

The data are presented for fatty acid in the unit % lipids and for lipid content in the unit g per 100g. Values are means of triplicate performance ± standard errors.

Table 15. (Table 3. Fatty acid composition in each treatment (cont.))

<table>
<thead>
<tr>
<th>UVR</th>
<th>vaccenic acid</th>
<th>oleic acid</th>
<th>linoleic acid</th>
<th>arachidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inner</td>
<td>outer</td>
<td>inner</td>
<td>outer</td>
</tr>
<tr>
<td>control</td>
<td>1.54 ± 0.03</td>
<td>1.48 ± 0.02</td>
<td>62.83 ± 0.43</td>
<td>62.40 ± 0.15</td>
</tr>
<tr>
<td>reflect</td>
<td>1.50 ± 0.02</td>
<td>1.46 ± 0.03</td>
<td>63.87 ± 0.10</td>
<td>62.40 ± 0.06</td>
</tr>
</tbody>
</table>

The data are presented for fatty acid in the unit % lipids and for lipid content in the unit g per 100g. Values are means of triplicate performance ± standard errors.
Table 16. (Table 4. Analysis of variance for tocopherols in UV and canopy treatment)

<table>
<thead>
<tr>
<th>source</th>
<th>df</th>
<th>sum of squares</th>
<th>mean square</th>
<th>v.r.</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV treatment</td>
<td>1</td>
<td>120.86</td>
<td>120.86</td>
<td>8.15</td>
<td>0.01</td>
</tr>
<tr>
<td>canopy position</td>
<td>1</td>
<td>13.91</td>
<td>13.91</td>
<td>0.94</td>
<td>0.344</td>
</tr>
<tr>
<td>uv treatment x canopy position</td>
<td>1</td>
<td>19.91</td>
<td>19.91</td>
<td>1.34</td>
<td>0.26</td>
</tr>
<tr>
<td>residual</td>
<td>20</td>
<td>296.54</td>
<td>14.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>γ-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV treatment</td>
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<td>0.00142</td>
<td>0.00142</td>
<td>0.12</td>
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<tr>
<td>canopy position</td>
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<td>0.01247</td>
<td>0.01247</td>
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<td>uv treatment x canopy position</td>
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<td>0.02327</td>
<td>1.92</td>
<td>0.181</td>
</tr>
<tr>
<td>residual</td>
<td>20</td>
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<td></td>
</tr>
<tr>
<td><strong>β-tocopherol</strong></td>
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<td>0.000429</td>
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<td>1</td>
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<td>0.015</td>
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<td>0.0000828</td>
<td>0.0000828</td>
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<td><strong>α-tocotrienol</strong></td>
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<td>0.012676</td>
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<td>0.308</td>
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<td>0.000714</td>
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<td>0.061493</td>
<td>0.003075</td>
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<td><strong>Total-Tocopherols</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>21.81</td>
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<td>20</td>
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Table 17. (Table 5. Analysis of variance for lipid and fatty acids in UV and canopy treatment)

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<th>Sum of Squares</th>
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<th>V.r.</th>
<th>P Value</th>
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<td><strong>Lipid Content</strong></td>
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<td>4.48</td>
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<td>0.693</td>
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<td>0.00009296</td>
<td>7.13</td>
<td>0.015</td>
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<td><strong>Palmitic Acid</strong></td>
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<tr>
<td>UV treatment</td>
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</tr>
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<td>uv treatment x canopy position</td>
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<td>0.179</td>
<td>5.79</td>
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<td>0.03089</td>
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<td></td>
</tr>
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<td><strong>Palmitoleic Acid</strong></td>
<td></td>
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</tr>
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<td>UV treatment</td>
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<td>0.0089935</td>
<td>0.0004497</td>
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<td></td>
</tr>
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<td><strong>Stearic Acid</strong></td>
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<td></td>
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</tr>
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<td>0.65</td>
</tr>
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<td>30.97</td>
<td>&lt;0.001</td>
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<td></td>
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<td></td>
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<td>0.041</td>
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<td>residual</td>
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<td>0.003271</td>
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<td><strong>Oleic Acid</strong></td>
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<tr>
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<td>1.6208</td>
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<td>5.433</td>
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<tr>
<td>UV treatment</td>
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<td>2.9775</td>
<td>24.08</td>
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<td><strong>Arachidic Acid</strong></td>
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</tr>
<tr>
<td>UV treatment</td>
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</tr>
</tbody>
</table>
CHAPTER SIX

Overall Discussion and Conclusion

This project, focusing on the major nutrients of almond, i.e. lipids and Vitamin E, investigated the factors most strongly influencing content and composition. The results are related to both industrial decision making and academic research.

At first, the investigation of regional and varietal difference provided a significant database for the Australian almond industry to manage almond plantation and marketing strategy in the global competition. The investigation within Australian growing regions showed that Willunga grown Nonpareil, Somerton and Johnston almonds had higher concentrations of linoleic acid than Riverland and North Adelaide Plains grown almonds. Willunga was very well known as an almond orchard area in the past, but nowadays, it has been largely turned into a vineyard region. This project gave evidence for Willunga to be an almond region once again. The results also showed Australia and California grown almonds had higher linoleic acid concentrations than Mediterranean grown almonds. Noticeably, Australian and Californian regions apply irrigation, but the Mediterranean almond orchards rely mostly on rainfall. It implies that to produce high nutritional value almonds, orchard management may adapt irrigation, as the result suggested that linoleic acid concentrations in almonds could be related to the water supply available to trees. However, the effect of drought on almond kernel nutrients in this project did not demonstrate a significant decrease of linoleic acid after deficit irrigation treatments, thus, we may consider soil type differences. It could spark a study on soil type pertaining to almond nutrients. Besides regional differences, varietal differences demonstrated that Carmel had the highest concentration of linoleic acid by 29.8 % compared
to other varieties. This indicates that the storage of Carmel almonds requires lower moisture content than other varieties, otherwise the kernels are susceptible to oxidation.

Apart from linoleic acid, Vitamin E is the most important nutrient of almond. The investigation showed that Guara almonds were rich in Vitamin E and also less affected by environmental difference, e.g. Spanish Guara and Australian Riverland Guara contained 21.5 and 18.9 mg/100g of Vitamin E, respectively. Following Guara, selection No. 13 was the second highest in Vitamin E concentration, at 15.6 mg/100g. Apparently, selection No. 13 has more nutritional standpoints than other new selections. Likewise, compared to Carmel, selection No. 13 had higher linoleic acid concentration by 26.3% and was in good balance regarding storage criteria with the O/L ratio at 2.37. Moreover, selection No. 13 has greater marketing potential due to its flavour characteristics and attractive appearance, therefore, among the new selections, selection No.13 is recommended to the industry for promotion.

Apart from investigating regional and varietal differences, this project specifically looked at almond orchard management improvement, including water supply, natural resource application and fruit maturation. First of all, given the drought issue has been addressed in a range of scientific areas, the significance of water saving solutions is considered as a priority for agricultural industry (Hamrouni et al. 2001; Baldini et al. 2002; Gigon et al. 2004; Karam et al. 2007; Laribi et al. 2009; Tang et al. 2011; Bettaieb et al. 2012). This project conducted an examination of drought effects on almond nutrients associated with deficit irrigation strategies. In regards to almond nutrient content, the results confirmed that moderate water deficiency, i.e. 85% of ET₀ water supply represents a feasible water saving solution in almond orchard irrigation, as no lipid or Vitamin E content was lost and fatty acid composition was not negatively changed. Specifically, the technique to apply the water saving solution is sustained deficit irrigation (SDI), rather than periodically conduct deficit irrigation which was
named regulated deficit irrigation (RDI) in this project. This study brings hope to almond growers in terms of financial benefits. A 15% water saving in almond orchards could substantially reduce input costs to the growers. In Australian inland climatic condition, one almond tree approximately requires 42,000 litres of water every year. If applying 85% deficit irrigation strategy, it will annually cut 6300 litres of water supply for one tree. Commonly, an almond orchard possesses 285 trees/ha, therefore, 1,795,500 litres of water will be saved in total per hectare per year. As well as almond orchards, this result could be transferable to other tree nut orchards.

Secondly, besides the drought issue, solar UV radiation in Australia has drawn more attention from scientists as well. High solar UV radiation is not beneficial for human health (Roy et al. 1995, Herman et al. 1998, Gies et al. 2003, 2004), however, this project observed increased solar UVR positively influenced almond lipids and tocopherols. The results showed 14% solar UVR enhancement, from reflection off white mat on the ground, had no effect on almond lipid content, but increased almond tocopherol concentration by 30%. Fatty acid composition was slightly changed: linoleic acid decreased by 2%, and oleic acid increased by 1.5%, so the O/L ratio was not altered. Overall, using white mat to reflect solar UVR could improve almond major nutrients, particularly in tocopherol concentration. This technique could be practiced in almond orchards to enhance the nutrition profile of almonds. Most importantly, the increase of UV radiation has to be moderate, because many studies demonstrated that high UV radiation caused damage to plants’ DNA (Costa et al. 2002, Gao and Zhang 2008). This project showed that environmental conditions, even if it is not good for humans directly, could have advantages for other aspects of human civilisation.

Relating to orchard management, lipid maturation and tocopherol accumulation during almond kernel development are the key points to control kernel quality. This study
demonstrated changes in almond kernel appearance with development as well as accumulation during lipid maturation and tocopherol accumulation. The rate of nutrient each growth stage after anthesis was also studied. The appearance of gelatine kernels indicated a slow phase of lipid build up, that is between 74 and 95 days post-anthesis only 0.38 g lipids /100 g kernel powder accumulated per day, whereas, the rate of tocopherol accumulation was at its highest point during this period, at 0.58 mg tocopherols/day in 100 g lipids. These results demonstrated almond tocopherol metabolism occurred in the early stage of almond lipid maturation and kernel ripening. From 95 days to 115 days, lipid content rapidly increased at 1.83 g lipids /100 g kernel powder /day, kernel appearance during this time was a creamy impression. Despite the fast rate of lipid maturation, tocopherol accumulation was rather slow at 0.09 mg tocopherol/day in 100 g lipids. This rate lasted till 135 days after anthesis even though lipid synthesis slowed down to 0.05 g lipids/100 g kernel powder/day. During this time, kernel appearance changed with the brown skin colour and the texture became more firm. Approaching kernel ripening, the rate of lipid maturation was slightly increased to 0.62 g lipids /100 g kernel powder /day, and was considered due to kernel moisture loss rather than lipid metabolic changes, meanwhile, at almost the same time, tocopherol accumulation increased at 0.28 mg tocopherol / day in 100 g lipids from 0.09 mg tocopherol / day in 100 g lipids. The reason has not been discovered. In general, almond tocopherol accumulation was in a positive correlation with the lipid maturation (R=0.864, p<0.0001). The related early stage from 95 days to 115 days after anthesis is the most important time to control almond kernel quality.

In conclusion, this project has significantly taken the industry’s interests into account, considering regional and varietal differences on almond composition for Australian almond plantations and production mapping. We recommend sustained deficit irrigation in almond orchards as part of water saving solutions, and suggest moderate solar UV radiation
application to improve the kernel quality. We have documented almond kernel lipid and Vitamin E development for the growers to refer to while fertilising, pruning and spraying. In short, this project brought viable outcomes to the industry and academic research alike.

In the future, the solar radiation application technique could be useful for almond canopy architecture projects, and the varietal differences would be helpful for market segmentation, using different types for different purposes. The results of sustained deficit irrigation will bring confidence for the growers to operate this water saving technique. The developmental stages of almond kernel lipid and Vitamin E could be useful for almond orchard management.
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