

Water use efficiency in Almonds (*Prunus dulcis* (Mill.) D. A. Webb)

Vahid Rahimi Eichi

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Abstract

Almond (*Prunus dulcis* (Mill) D. A. Webb) is a nut tree in the family Rosaceae, which compared to other nut crops, grown in Mediterranean climates, is relatively drought resistant. Due to the lack of, or high cost of water, almond growers are more inclined to improve gross production water use efficiency (WUE) by adopting water saving irrigation strategies. To this aim, the sensitivity and accuracy of different water status indicators need to be compared to design a suitable irrigation schedule. Meanwhile, instantaneous water use efficiency (WUE_i) that is a measure made at the leaf scale can also be used as a criterion for estimating WUE in breeding programs.

To study the effects of different deficit irrigation strategies, sustained and regulated deficit irrigations (SDI and RDI) were applied on almond trees for two consecutive seasons (2009-2010 and 2010-2011). Five levels of water amount were applied; namely, 55, 70, 85, 100 and 120% ET_c. Kernel yield, midday stem water potential (MSWP), stomatal conductance (g_s), increment in trunk circumference (ΔTC) and carbon isotope discrimination (Δ¹³C) were measured for both seasons. Results obtained in the 2009-2010 season showed that regardless of irrigation strategy, kernel yield was reduced in 70% ET_c of irrigation or less. Meanwhile kernel yield, WUE and water status indicators in this season were more sensitive to the quantity of water applied rather than to the deficit strategy (SDI or RDI). However, kernel yield was slightly lower in RDI 70% ET_c compared to SDI 70% ET_c treatments.

Although, there were high correlations between all water status indicators and the amount of water applied, g_s and Δ¹³C showed lower sensitivity towards water deficit compared to MSWP and ΔTC, implying an anisohydric behaviour of almond trees. Meanwhile, in the first season, the observed correlation coefficients between kernel yield and ΔTC were lower than those of other water status indicators: MSWP ≈ g_s ≈ Δ¹³C > ΔTC. In addition, there was only a moderate correlation (R² = 0.61) between Δ¹³C and WUE in the first season indicating that Δ¹³C may not be a reliable indicator of changes in WUE in almond trees. In the 2010-2011 season, there were no significant differences in kernel yields and water status indicators

between different treatments. It was probably due to the humid weather and frequent rain in the second season that negated the effects of deficit irrigation on almond trees.

To study the WUE_i in different genotypes, g_s and assimilation rate (A) in 5 mixed crosses of almond were examined. The significant correlations between g_s , A and internal concentration of CO_2 (C_i) indicated that A was probably limited by both stomatal and non-stomatal parameters that might be affected by genotype variations. Mesophyll anatomy and g_s between three almond varieties (Nonpareil, Carmel and Masbovera) were also compared. The results demonstrated that the post-venous hydraulic distance D_m and the density of mesophyll cells might indirectly affect g_s .

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List of Abbreviations

A	assimilation rate per unit of leaf area ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
C_a	external CO_2
C_i	Internal CO_2
D_m	post-venous hydraulic distance
E	transpiration rate per unit of leaf area ($\text{mmol m}^{-2} \text{s}^{-1}$)
E_{pan}	class A evaporation pan
ET_c	potential crop evapotranspiration
ET_o	reference crop evapotranspiration
g_c	stomatal conductance to CO_2 ($\text{mmol m}^{-2} \text{s}^{-1}$)
g_m	mesophyll conductance ($\text{mmol m}^{-2} \text{s}^{-1}$)
g_s	stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$)
g_w	stomatal conductance to water vapour ($\text{mmol m}^{-2} \text{s}^{-1}$)
HCFM	hydraulic conductance flow meter
k_{leaf}	leaf hydraulic conductance
K_c	crop coefficient
KF	kernel fraction
KY	kernel yield (t ha^{-1})
L_1	leaf hydraulic conductance normalized to leaf area ($\text{kg s}^{-1} \text{mpa}^{-1} \text{s}^{-2}$)
MDB	Murray-Darling River Basin
MSWP	midday stem water potential (Mpa)
PPFD	photosynthetic photon flux density $\mu\text{mol m}^{-2} \text{s}^{-1}$
RDI	regulated deficit irrigation
SDI	sustained deficit irrigation
VPD	vapour pressure deficit
W_a	external water vapour

W_i	internal water vapour
WUE	water use efficiency or gross production water use efficiency (kg mm^{-1})
WUE_i	instantaneous water use efficiency ($\mu\text{molCO}_2 \text{ mmol}^{-1} \text{H}_2\text{O}$)
$\Delta^{13}\text{C}$	carbon isotope discrimination
δ_a	carbon isotope composition in atmosphere
δ_p	carbon isotope composition in plant tissue
$\delta^{13}\text{C}$	isotope discrimination for carbon 13
ΔTC	increment in trunk circumference (mm)

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CHAPTER ONE

General introduction and literature review

1.1 Almonds in the world and in Australia

Almond (*Prunus dulcis* (Mill.) D. A. Webb; syn *P. amygdalus* Batsch) is a nut tree in the family Rosaceae, which has been farmed by humans for thousands of years. Two incompatible hypotheses are suggested about the origin and also the ancestors of almonds. In 1999, Ladizinsky proposed the first hypothesis based on the concept that almonds originated in the Middle East through domestication of *P. fenziiana* (Ladizinsky, 1999); but in 2005 other researchers indicated that the Mediterranean region was the original location for almond species. They also presented *P. webbii* as the most likely ancestor of almond (Socias i Company, 2004).

Owing to recent orchard plantings for increasing production of almond in the majority of leading producer countries, the global output of almond cultivation has improved in recent years. In 2012, United States (80.1%), Spain (4.8%), Australia (4.3%) and Turkey (1.3%) are the four major almonds producing countries (Figure 1.1) (The Almond Board of Australia, 2012).

In 1997, the Australian almond breeding program commenced at the University of Adelaide. The primary aim of this program is breeding and production of new types of scions and rootstocks that are more suitable for the Australian climate (Sedgley and Collins, 2002). However, due to new changes in tax rates that affected managed investment patterns, in addition to limitations in water allocation as a consequence of drought, the annual rate of almond plantings in Australia has been considerably reduced during recent years (Figure 1.2). The most common almond varieties grown in Australia are Nonpareil, Carmel and Price, which are commercially cultivated in three states comprised of Victoria, South Australia and New South Wales (Figure 1.3) (The Almond Board of Australia, 2012).

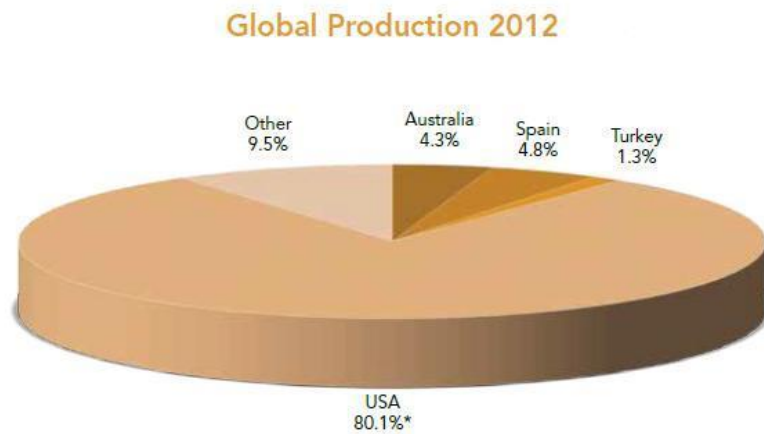


Figure 1.1 Australia's share of global almond production in 2012
(The Almond Board of Australia, 2012)

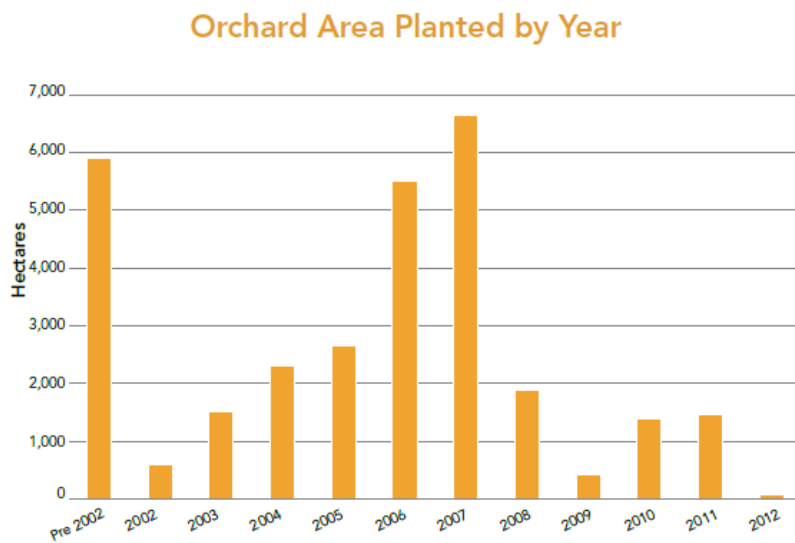


Figure 1.2 The estimated areas of almond plantings in Australia
(The Almond Board of Australia, 2012)

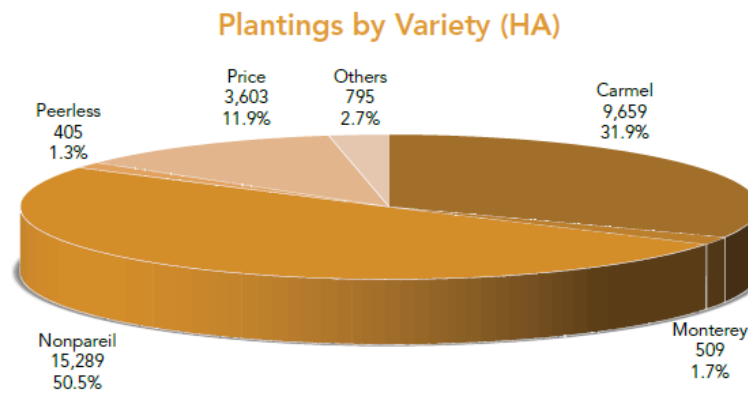


Figure 1.3 Almond production in Australia by variety in 2012
(The Almond Board of Australia, 2012)

1.1.1 Almond irrigation in Australia

Approximately 40% of all Australian farms are located in Murray-Darling River Basin (MDB) which is regarded as the most productive region for agriculture in Australia (Figure 1.4) (Bryan and Marvanek, 2004, Brown, 2011). In this regard, MDB annually produces about 15 billion (40%) of the gross value of Australian agricultural production. However, MDB accounts for 66 per cent of Australia's agricultural water consumption (Brown, 2011). The "Lower Murray" is well known for its high value of irrigated agriculture, therefore, reduced irrigation is considered as a massive problem for this area. This production area includes Sunraysia, Riverland areas and the Lower Lakes region. The overall amount of water consumption in each irrigation season includes the irrigation water plus effective rainfall. Effective rainfall is that part of rain water that remains in the root zone and can be used by plants. Water lost by deep percolation, or surface water runoff is not included in effective rainfall (Dastane, 1978). During recent decades, increasing water demands coupled with the reduction of overall rainfall in the upper catchment of Murray River has



Figure 1.4 Murray-Darling River Basin

Source: <http://ramblingsdc.net/Australia/MurrayDarling.htm>

limited water allocation for Lower Murray region. Bearing in mind that this area produces the majority of commercial Australian almonds, finding more efficient and effective deficit irrigation methods for almond orchards is considered as a high research priority (Sommer et al., 2010).

1.2 Water restrictions and solutions

In the recent decade, natural flows into the MDB considerably reduced because of reduced precipitation. As a consequence, in 2006-2007 the levels of water flowing in the Murray River was the lowest in 115 years of record keeping (Murray-Darling Basin Commission, 2008).

Because of the tremendous increase in human population, more water resources will be needed in the future. In this regard, the current level of water consumption by agriculture, which accounts for approximately 80% of the available water resources, cannot be sustainable (Condon et al., 2004). Water can be lost through two different pathways from the soil: First is

evaporation and the other is penetrating water to the lower levels of soil. The combination of soil evaporation and plant transpiration is called evapotranspiration (ET).

In some types of soils, irrigating plants increases the concentration of salt in the root zone, therefore moving water to the lower horizons prevents the toxic effects of extreme salt concentration in this area (Abbott and El Quosy, 1996). Bearing in mind that transpiration from leaves is coupled to photosynthesis, severe limitation of ET can have deleterious effects on productivity (Tanner and Sinclair, 1983, Steduto et al., 2007). Therefore, it is important to define a suitable irrigation schedule that consumes minimum amounts of water for producing optimum potential yield (Goldhamer et al., 2006). In this regard, several studies showed promising results for improving irrigation efficiency by using new irrigation methods in irrigated conditions (Cantero-Martinez et al., 2007, Kirda et al., 2007, Hamdy et al., 2003, Saeed et al., 2008).

1.3 Effects of drought stress on Almonds

Almond is regarded as tolerant to water deficit conditions (Feres and Goldhamer, 1990, Torrecillas et al., 1996); however, irrigation scheduling has considerable effects on improving the quality of nuts (Castel and Feres, 1982, Nanos et al., 2002). Teviotdale et al., (2001) reported that moderate drought stress at kernel filling stage decreases some fungal disease, e.g. spur die back. On the other hand, applying deficit irrigation regimes during the growth period reduces both size and weight of kernels (Teviotdale et al., 2001, Goldhamer and Smith, 1995, Goldhamer and Salinas, 2000, Esparza et al., 2001). Furthermore, there are some reports of increasing the infestation of spider mite under water stress conditions for almond trees (Youngman and Barnes, 1986).

Previous experiments showed that the sensitivity of almond orchards towards water stress is higher during post-harvest period compared to the kernel filling stage. In fact, applying deficit irrigation at this period limits growth in the following season, which consequently reduces the

fruiting positions in trees (Prichard et al., 1992 , Esparza et al., 2001, Goldhamer and Viveros, 2000). Meanwhile, the differentiation of reproductive buds that happens at post-harvest stage can be disrupted by water stress (Goldhamer and Smith, 1995, Goldhamer, 1996 , Goldhamer and Salinas, 2000, Esparza et al., 2001).

Unlike peach trees that are highly resistant toward drought stress at kernel filling period (Girona et al., 2003, Li et al., 1989), severe water deficit at kernel filling stage significantly reduces the kernel yield in almonds. The reason is that in most almond cultivars, kernel filling occurs during the summer when evaporative demand is at its maximum level; thereby, severe water deficit can disrupt growth in orchards (Girona, 1992, Kester et al., 1996). However, under mild to moderate drought stress, no significant loss of kernel yield has been observed (Goldhamer and Viveros, 2000, Esparza et al., 2001, Girona et al., 1997).

1.4 Water use efficiency

Due to the lack of water resources and the high cost of water, it is more profitable for almond growers to use more water efficient irrigation strategies than the conventional methods (Goldhamer et al., 2006, Fereres and Auxiliadora Soriano, 2007). Practically, there are two basic definitions of water use efficiency (WUE): WUE of productivity (Equations 1.1, 1.2 and 1.3) and photosynthetic WUE (Equations 1.4, 1.5) (Purcell and Associates, 1999, Martin and Thorstenson, 1988, Xu et al., 2010). The first three definitions are mostly applied for industrial estimations and indicate the WUE of the whole plant; whereas, instantaneous WUE (WUE_i), which is a measure made at the leaf scale, represents the ratio of assimilation rates (A) to transpiration (E) (Van den Boogard et al., 1995, Purcell and Associates, 1999).

$$\text{Crop Water Use Index} = \text{Total Product (kg)} / \text{Transpiration (mm)}$$

(Equation 1.1)

$$\text{Irrigation Water Use Index} = \text{Total Product (kg)} / \text{Irrigation Water supplied to farm gate (ML)}$$

(Equation 1.2)

Gross Production Water Use Efficiency = Total Product (kg) / Total Water used on farm
(mm)

(Equation 1.3)

Transpiration Water Use Efficiency = Total biomass (kg) / Transpired Water (mm)

(Equation 1.4)

Instantaneous Water-Use Efficiency $WUE_i = A (\mu\text{mol CO}_2) / E (\text{mmole H}_2\text{O})$

A = photosynthesis rate

E = transpiration rate

(Equation 1.5)

Stomatal closure that is an immediate consequence of water deficiency in plants, increases WUE by limiting water loss diffusion to a greater extent than CO_2 uptake. Opening the stomatal aperture which reduces the stomatal resistance by a factor of two, increases the concentration of intercellular CO_2 (C_i) from 250 to 330 ppm (Heldt and Piechulla, 2011). Although, the rate of carboxylation would be improved by increasing C_i , however, in this condition, water loss will be doubled due to the reduction of the stomatal diffusion resistance (Farquhar and Sharkey, 1982, Heldt and Piechulla, 2011). More than 90% of the water that is taken up by roots is lost through the stomata without involvement in any biochemical processes (Morison et al., 2008). Because of the considerably higher driving force for water vapour loss than that for CO_2 absorption into the leaf, crop plants mostly lose high volumes of water in order to uptake, comparatively, modest amounts of CO_2 (Ort et al., 1994, Chaves et al., 2002). In fact, compared to CO_2 uptake, H_2O efflux is more dependent on stomatal resistance. Stomatal aperture regulations help to optimize CO_2 uptake without losing excessive amounts of water (Zelitch, 1969, Heldt and Piechulla, 2011). Nevertheless, continued stomatal closure ultimately reduces carbon assimilation and photosynthesis (Farquhar and Sharkey, 1982).

1.4.1 Improving the efficiency of deficit irrigation strategies

Almond trees are relatively resistant to drought stress compared to other nut crops, grown in Mediterranean climates, however, summer droughts in such areas considerably decrease productivity (Castel and Fereres, 1982, Hutmacher et al., 1993). Thus irrigation is an important component of almond production, which is 10 fold higher on an area basis compared to that of traditional non-irrigated almond cultivation (Girona, 1992). Therefore, the prevalence of irrigated cultivation has led to an almost complete abandonment of traditional non-irrigated systems (Girona et al., 2005).

Generally in crops, there is a linear correlation between water applied and yield, but it only continues until half the amount of water required for full irrigation is given (full irrigation =100% of potential crop evapotranspiration) (Doorenbos et al., 1979, Hargreaves and Samani, 1984). Above this level, the probability of water loss increases due to deep percolation (Peri et al., 1979, Norum et al., 1979) and evapotranspiration (Hanks and Hill, 1980, Carvallo et al., 1975) with no increase in yield. In other words, the irrigation water use index reduces as water applied approaches full irrigation (100% ET_c). Related studies on various fruit trees such as almonds (Goldhamer and Salinas, 2000), pistachios (Goldhamer and Beede, 2004), citrus (Domingo et al., 1996, Gonzalez-Altozano and Castel, 1999), apple (Ebel et al., 1995), apricot (Ruiz-Sanchez et al., 2000), wine grapes (Bravdo and Naor, 1996, McCarthy et al., 2002) and olive (Moriani et al., 2003) confirm that full irrigation (100% ET_c) cannot necessarily improve irrigation efficiency (Fereres and Evans, 2006, Fereres and Soriano, 2007).

It is possible to increase WUE by using deficit irrigation (DI) in arid and semi arid areas. Previous reports showed that despite the relative decrease in the vegetative growth of peach orchards grown under DI conditions, no significant yield losses were observed (Mitchell and Chalmers, 1982, Chalmers et al., 1981). When a constant level of DI is applied throughout the whole growing season it is commonly referred to as sustained deficit irrigation (SDI). Conversely, when DI is not applied continuously but is restricted to defined periods within a

season, e.g. pre-harvest, it is often referred to as regulated deficit irrigation (RDI). Related studies on almond irrigation implied that applying RDI can improve WUE and crop productivity in water limited areas (Goldhamer et al., 2006, Goldhamer and Viveros, 2000, Boyer, 1996). Alegre et al (2000) reported that in olive trees, applying RDI advanced ripening dates, which consequently increased the volume of extracted oil. Meanwhile they found no significant reduction of oil yield in RDI compared to control treatments (Alegre et al., 2000). Applying RDI in peach and pear trees reduced irrigation by approximately 30% with no significant decrease in yields (Kriedemann and Goodwin, 2002). In a similar experiment on the responses of pistachio trees to applying RDI during the early stages of fruit development, a slow recovery during kernel filling stage was reported. In this regard, Guerrero et al (2006) recommended using RDI by applying full irrigation (100% ET_c) before the commencement of kernel filling period in pistachio trees (Guerrero et al., 2006). Application of suitable DI strategies has also been shown to decrease the detrimental effects of irrigation on the environment by reducing water leaching and water extraction from rivers (Smith et al., 1996, Verma, 1986, Wichelns and Oster, 2006).

1.4.2 Carbon Isotope Discrimination

For the first time Nier and Gulbransen in 1939 reported the natural difference between the amounts of ¹²C (light isotope) and ¹³C (heavy isotope) in the atmosphere (Nier and Gulbransen, 1939). Wickman (1952) observed the same differences within plant tissues (Wickman, 1952).

The natural fraction of carbon isotope is dominated by ¹²C, constituting the majority (98.9 %) of total atmospheric carbon, compared to 1.1% for ¹³C. Carbon isotope fractionation is defined as the discrimination by the plant between heavy and light carbon isotopes. The main factor contributing to carbon isotope fractionation is the discrimination by Rubisco (Ribulose-1, 5-Bisphosphate carboxylase /oxygenase) between ¹²CO₂ and ¹³CO₂ (Lanigan et al., 2008,

Farquhar et al., 1989). Because of the relatively heavy mass, the diffusion rate of $^{13}\text{CO}_2$ through the stomata is 4.4‰ less than that of $^{12}\text{CO}_2$. Meanwhile, there is a discrimination of 29‰ against ^{13}C isotope ($\Delta^{13}\text{C}$) for the reactions regulated by key photosynthetic enzymes including Rubisco (Farquhar et al., 1989).

Stomatal restrictions, as a response to water deficit, decrease the ratio of internal to external CO_2 (C_i/C_a). Stomatal closure decreases the flux of CO_2 into the leaf interior, which subsequently reduces C_i/C_a values. Since the tendency for $^{12}\text{CO}_2$ in carboxylation is higher than that of $^{13}\text{CO}_2$, therefore the concentration of $^{13}\text{CO}_2$ molecules in intercellular spaces increases after stomatal closure. Thus, lower C_i/C_a ratio leads to reduced fractionation because it drives Rubisco to fix higher proportions of $^{13}\text{CO}_2$ (Figure 1.5A) (Farquhar et al., 1989). In contrast, after the stomata open, CO_2 diffuses easily through the stomata into the intercellular spaces, which gives more options for Rubisco to discriminate against $^{13}\text{CO}_2$. In other words, stomatal closure reduces the selective discrimination against ^{13}C during carboxylation (Farquhar et al., 1982). In fact, $\Delta^{13}\text{C}$ values demonstrate the variations in leaf diffusive resistance between different water stress levels (Farquhar et al., 1989). Based on this concept, Farquhar et al. (1989) reported that $\Delta^{13}\text{C}$ is negatively correlated with instantaneous water use efficiency (WUE_i) that is a component of transpiration water use efficiency. Under constant water vapour pressure, transpiration water use efficiency, which is defined as the whole biomass or yield produced per unit of water loss by transpiration (Equation 1.4), shows a negative indirect correlation with $\Delta^{13}\text{C}$ (Farquhar et al., 1989). Although some experiments showed that water use efficiency at leaf level may not always represent the whole plant water use efficiency, there are some reports implying close correlations between $\Delta^{13}\text{C}$ and whole plant water use efficiency, e.g. gross production water use efficiency (Figure 1.5B) (Lambers et al., 2008, Condon et al., 2004).

Mostly, the accumulated carbon in plants is absorbed over a considerable period of time under different climate conditions. Therefore, in contrast with gas exchange measurements, which only monitor instantaneous values, the long term metabolic and stress history of plants can be

recorded by measuring $\Delta^{13}\text{C}$ (Ehleringer et al., 1992, Johnson et al., 1990). Related studies on different types of plants, e.g. grapevine (Gibberd et al., 2001), crested wheat grass, Altai wild rye, orchard grass, tall fescue and perennial ryegrass confirm the importance of $\Delta^{13}\text{C}$ values for estimating WUE (Ebdon et al., 1998).

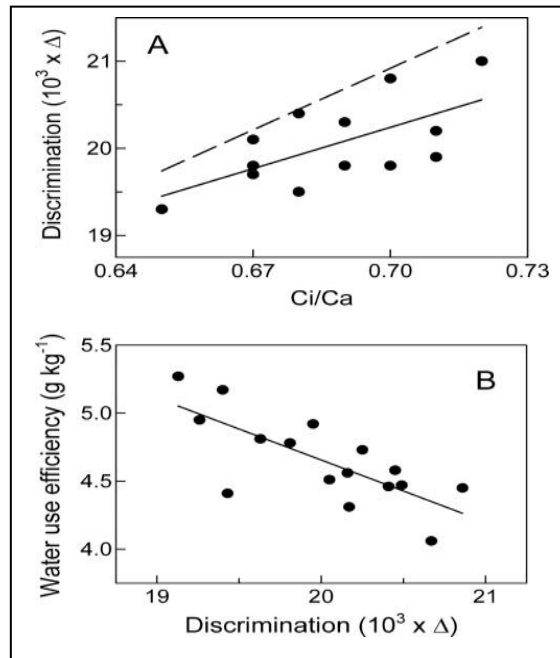


Figure 1.5 Relationships between $\Delta^{13}\text{C}$ and C_i / C_a (A) and between $\Delta^{13}\text{C}$ and WUE (B) in the leaves of wheat.

Source: Condon A. G. et al. (2004)

CHAPTER TWO

Comparison of different water status indicators in almond (*Prunus dulcis*) trees grown under two deficit irrigation strategies.

2.1 Introduction

Despite the relative tolerance of almonds to water deficit (Stewart and Nielsen, 1990), irrigation scheduling is still important for improving yield production in almond orchards (Castel and Fereres, 1982). Irrigation scheduling is the growers' decision process regarding the right time and the right quantity of water required for irrigation (Heermann, 1996, Pereira, 1999). Theoretically, irrigation scheduling for efficient water use requires correct assessment of the daily crop evapotranspiration (ET_c) (Allen et al., 1998), a clear estimation of plant water status (Campbell and Mulla, 1990, Hsiao, 1990), an understanding of the soil water holding properties (Hedley et al., 2010), and the effects of water deficit on yield (Heermann, 1996, Pereira, 1999). The level, beyond which irrigation is necessary to avoid the detrimental effects of water deficit on a plant, is defined as the threshold of drought tolerance. Applying irrigation levels beneath the threshold of drought tolerance reduces both quantity and quality of yield. Therefore, a precise estimation of the threshold of tolerance towards water deficit can help to design suitable irrigation schedules in almond trees (Jones, 2004, Romero et al., 2010). However, the threshold of drought tolerance in plants is not constant under different types of soil texture (Schwankl et al., 1999). Soil texture has unique effects on both water holding capacity and water movement through the soil (Kramer and Boyer, 1995). Soil moisture can be measured by two different approaches: the volumetric soil measure (quantitative method), which shows how much water exists in the soil, and soil water tension

(qualitative method) that represents how easily water can be absorbed by plant roots. In fact, soil water tension shows the levels of adherence between water and soil particles. However, the relationship between volumetric soil and soil tension is not linear and changes with different types and depths of soil (Boulding and Ginn, 2010, Campbell-Clause, 2007). Despite numerous studies, still there is not a completely reliable criterion for designing a precise irrigation schedule in tree crops under high frequency irrigation (Fernandez et al., 2002, Intrigliolo and Castel, 2006a, Nortes et al., 2005, Remorini and Massai, 2003).

In this experiment “gross production water use efficiency WUE” (Equation 1.3) was used for the definition of water use efficiency, which is defined as the total product of kernel (kg) per total water applied (including rainfall) (mm) (Purcell and Associates, 1999). The effects of different irrigation treatments on WUE give references for irrigation management (Zhang et al., 2008). Besides crop and soil characteristics, applying different irrigation practices, including sustained and regulated deficit irrigations, partial root zone drying (PRD) and subsurface drip irrigation, can also affect WUE (Kirida, 2002).

A crucial issue in agricultural management is to find a reliable method for predicting the variations of yield and WUE (Moa et al., 2005). In this regard, previous experiments confirmed that strategic management of irrigation can improve WUE and yield in crops (Botwright Acuna et al., 2010). Achieving an effective yield management requires a precise prediction of yield variations under different water schedules (Bornn and Zidek, 2012). To this aim, evaluating different predictive criteria for yield and WUE can help to design more efficient irrigation schemes in water limited conditions. For instance, plant water status can provide essential knowledge for irrigation management (Nortes et al., 2005). Plant water status affects crop productivity mostly by controlling growth and development. The majority of principal physiological processes e.g. cell enlargement, photosynthesis and stomatal behaviour, are directly or indirectly driven by plant water contents. Water status of plants can be controlled by atmospheric evaporative demand, soil water content, root capacities to

uptake water and stomatal behaviours; therefore plant water status represents the interactions between atmosphere, plant and soil (Gimenez et al., 2005).

Since water limitations during particular stages of growth periods, e.g. flowering stage, significantly reduces yield, therefore, early detection of any water deficit, even if it is applied for a short period, can help to prevent yield losses (Suárez et al., 2012). Those indices which respond relatively quickly to water status variations, can precisely record even short term water deficit periods. In this regard, Ben-Gal, et al (2010) noted both midday stem water potential (MSWP) and stomatal conductance (g_s) as suitable indicators for recording short term severe water stresses in olive trees (Ben-Gal et al., 2010).

Previous studies recommended MSWP for monitoring both moderate and severe plant water deficits, as the most discriminating and comprehensive index in grape vines (Chone et al., 2001) and plum trees (Shackel et al., 1997). On the other hand, midday leaf water potential (LWP) is not regarded as an accurate indicator of water stress; mainly because LWP is more susceptible to local climate fluctuations (Chone et al., 2001). Moreover, transpiration rate, stomatal conductance g_s , soil moisture content and hydraulic conductivity in the trunk are more correlated with MSWP compared to LWP (Chone et al., 2001, McCutchan and Shackel, 1992). Contrarily, Intrigliolo and Castel (2006) reported that LWP showed higher sensitivity to different irrigation treatments in comparison with MSWP. According to their results, midday stomatal conductance was more correlated with LWP than MSWP (Intrigliolo and Castel, 2006b). However, measuring water potential can represent a snapshot but not a continuous overview of the plant water status (Ferreira and Goldhamer, 2003).

Due to the high sensitivity of g_s to water stress, it is also a suitable indicator of plant water status (Garcia-Tejero et al., 2011, Medrano et al., 2002). Klein et al. (2001) revealed the importance of stomatal behaviour as a reversible response to moderate water deficit during the harvest period in almond trees (Klein et al., 2001). Both g_s and MSWP are therefore widely used by plant scientists as effective indicators for irrigation management (Doltra et al., 2007, Naor, 2000, Flexas et al., 2002, Nortes et al., 2005, Rosati et al., 2006). Related studies on

almond trees introduced the daily and seasonal variations of trunk circumference (ΔTC) as a possible substitute for MSWP (Goldhamer and Fereres, 2004, Nortes et al., 2005).

Farquhar and Richards (1984) suggested the practical use of $\Delta^{13}C$ for the estimation of WUE (Farquhar and Richards, 1984). Meanwhile, there are some reports that $\Delta^{13}C$ can be used for evaluating water status in plants (Gaudillere et al., 2002). The strong relationship between $\Delta^{13}C$ and C_i/C_a is confirmed for different time scales: relatively instantaneous during gas exchange measurements (Evans et al., 1986, Ouerghi et al., 2000), days for recently produced carbohydrates (Brugnoli et al., 1988) and weeks to months for leaves (Farquhar et al., 1989).

Bearing in mind that collecting extensive series of data throughout the season for measuring $\Delta^{13}C$ is difficult, accordingly, there are some promising breeding practises demonstrating the effectual role of $\Delta^{13}C$ for improving water use efficiency (Raiabi et al., 2009, Condon et al., 2006).

For minimizing the devastating effects of water deficit on fruit trees, deficit irrigation can be applied during periods in which trees are relatively invulnerable to water deficit. Therefore, detecting such drought tolerant periods can be useful for scheduling more efficient RDI strategies (Mitchell et al., 1989, Fereres and Goldhamer, 1990). For instance, in some RDI studies, yield parameters did not show significant reduction under mild water deficit (Romero et al., 2004a, Romero et al., 2004b). In this respect, besides SDI treatments, RDI were also applied in this experiment to investigate the impacts of different water deficit periods on yield and plant water status in almond orchards. The primary goal was to determine the best irrigation strategy for improving WUE in almonds. Similar to the study conducted by Goldhamer et al. (2006), in this experiment the impacts of SDI and RDI on kernel yield were examined. Meanwhile, in this study the effects of SDI and RDI on different water status indicators were measured; whereas Goldhamer et al. (2006) emphasised on determining the impacts of SDI and RDI on harvestability, insect damage and nut quality. However, despite Goldhamer et al. (2006) who measured LWP in their experiments, in this study MSWP, g_s , ΔTC and $\Delta^{13}C$ were measured as water status indicators (Goldhamer et al., 2006). Since Goldhamer et al. (2006) reported the negative effects of post harvest deficit irrigation on yield

in the following season (Goldhamer et al., 2006, Goldhamer and Viveros, 2000), no such deficit treatment was applied during post harvest period in this study.

The other objective was to evaluate the strength of correlation between both physical (MSWP, ΔTC and g_s) and biochemical ($\Delta^{13}C$) water stress indicators with kernel yield under field conditions. Meanwhile, the possibility of using $\Delta^{13}C$ as a surrogate selection criterion in breeding programs for the prediction of yield and WUE was examined.

2.2 Materials & Method

2.2.1 Site

The field trial was established at the end of the 2008-2009 season and field measurements were carried out during the 2009-2010 and the 2010-2011 seasons. The trial was located near Lake Powell in North West Victoria (Lat: -34.706° S and Long: 142.874° E). The orchard was established in 2004. The soil texture was uniform across the site ranging from a fine sandy loam to a sandy loam or loamy sand with increasing depth. Alternate almond rows (*P. dulcis* Mill.) of the varieties Nonpareil and Carmel were planted in a north-south direction at a tree distance of 4.65 m within and 7.25 m between the rows. The rootstock was Nemaguard and the experimental area comprised 5.2 ha. Trees blossomed in mid August in both seasons and were harvested using commercial tree shakers on 17 February 2010 and 2 March 2011.

2.2.2 Irrigation treatments

Treatments included: (1) control: standard practice irrigation (100% of potential crop evapotranspiration ET_c over the entire season) during the whole growing season; (2) sustained deficit-irrigation (SDI): applying deficit irrigation throughout the irrigation season at 55%, 70% and 85% ET_c (3) regulated deficit irrigation (RDI): deficit irrigation (50%, 70% and 85% ET_c) applied pre but not post-harvest during selected periods, (4) wet irrigation: 120% of ET_c applied throughout the season to assess the potential for root zone drainage. Therefore, in total there were 8 irrigation treatments with 6 replicates (Figures 2.1, 2.2 and Table 2.1). There was an irrigation control unit that automatically applied water for each treatment by turning off/on the watering process with hourly pulses. By way of explanation, irrigation was applied for one hour and turned off for the subsequent hour, then repeated until the full water requirement was met. Therefore, different irrigation rates were achieved by different irrigation periods. The volume of water applied, per day, was measured by using a flow meter for each irrigation treatment. The irrigation hours for each day and each treatment were estimated from the long

term evaporation records or the short term forecasts, after adjusting for the previous day's irrigation tally (previous day's evaporation - previous day's irrigation application).

Table 2.1 Timing of sustained (SDI) and regulated deficit irrigation (RDI), control and 'wet' irrigation treatments applied at Lake Powell for the 2009-2010 and 2010-2011 seasons. Below the line shows post harvest period.

Period	wet	con.	rdi. 85	sdi. 85	rdi.70	sdi.70	rdi.55	sdi.55
Aug 15-31	120	100	100	85	100	70	100	55
Sep 01-10	120	100	100	85	100	70	100	55
Sep 11-30	120	100	100	85	100	70	50	55
Oct 01-31	120	100	100	85	100	70	50	55
Nov 01-12	120	100	100	85	100	70	50	55
Nov 13-30	120	100	100	85	50	70	50	55
Dec 01-31	120	100	100	85	50	70	50	55
Jan 01-10	120	100	100	85	50	70	50	55
Jan 10-31	120	100	50	85	50	70	50	55
Feb 01-15	120	100	50	85	50	70	50	55
Feb 01-15	120	100	100	85	100	70	50	55
Feb 16-28	120	100	100	85	100	70	100	55
Mar 01-31	120	100	100	85	100	70	100	55
Apr 01-30	120	100	100	85	100	70	100	55
May 01-31	120	100	100	85	100	70	100	55

Effective rainfall was calculated as 50% of the rainfall equal to or above 12 mm during a period of 24 hours (Dastane, 1978). The estimation of daily ET_c were based on the daily readings from a class A evaporation pan (E_{pan}) located near the experimental site and were derived by multiplying the reference crop evapotranspiration (ET_o) by crop coefficient (K_c) (Equation 2.1). ET_o was recorded with a nearby automatic weather station and calculated based on Walter et al. (2000) methodology (Walter et al., 2000). The final project report conducted by Sommer (2012) describes the procedure used to determine K_c (Sommer, 2012).

$$ET_c = K_c \times ET_o \quad \text{Equation 2.1}$$

Fertigation was applied according to the current industry standard based on results from the almond optimisation trial. All treatments received the same amount of nutrients injected into the final irrigation pulse of the day (Sommer, 2012, The Almond Board of Australia, 2011).

2.2.3 Plant measurements

After harvest, nuts were left to dry on the ground until the hull moisture reached approximately 14%, 9 days in 2010 and 16 days in 2011. The 14% moisture is an approximate value and serves as a guide for when the nuts are ready to be picked up from the ground. Nuts were regularly sampled by the company using a moisture meter to determine the value. After sweeping the nuts of each harvested plot, comprising the 4 central trees, into windrows they were picked up into bulk bags. Then, bags were weighed and 3 kg sub-samples were collected for kernel yield. The sub-samples were dried to a constant weight and hull, shell and kernel dry weights of the sub-sample were determined.

Leaf g_s and MSWP were recorded fortnightly. Data were collected from 15 September 2009 to 30 March 2010 in the first season and from 29 September 2010 to 1 March 2011 in the subsequent season. g_s measurements were taken using a leaf porometer (Decagon, model SC). Measurements were recorded between approx. 0900 and 1500 h solar time. The operator always moved from plot to plot within a replicate and every time recorded measurements from two leaves per plot of each of two central trees. Each of the six replicates was visited successively resulting in 96 readings for one complete round of measurements (2 trees/plot x 8 treatment plots x 6 replicates). Throughout the course of a day this operation was repeated 3 times resulting in a total of 288 readings (96 x 3).

In sunny conditions measurements were always recorded from fully sun-exposed leaves. In overcast conditions such leaves were chosen that would be most probably exposed in full sun. MSWP was monitored using a Scholander pressure chamber (Plant Water Status Console 3005 series, Soil Moisture Equipment Corp., Santa Barbara, CA) (Ritchie and Hinckley,

1975). Data were recorded after one or two hours of enclosing an inner canopy leaf in a foil laminate bag (PMS Instrument Company, Albany OR). On each measuring date two leaves from each plot of the three western most blocks were tested.

ΔTC were monitored by measuring trunk girth in 24 replicates for 8 treatments. The measurements were taken for 2 consecutive years at the commencement (October) and again at the end (May) of the irrigation seasons.

Heterotrophic tissues are generally more enriched in ^{13}C compared to photosynthetic tissues (Cernusak et al., 2009); therefore in this study the $\Delta^{13}C$ of the shells, which constitute the woody parts of almond fruits, were measured. Depending on age and position, leaves can be considered as either sink or source organs during different stages of their life. Bearing in mind that $\Delta^{13}C$ values are different between source and sink tissues, using shells for measuring $\Delta^{13}C$ can minimize the possible differences in $\Delta^{13}C$ between different tissues (Cui et al., 2009, Cernusak et al., 2009). Moreover, fruit yield is mostly (70%) affected by environmental factors (Jaggi et al., 2002), thus $\Delta^{13}C$ values of fruits can represent the environmental conditions, especially water scarcity, during the growth season, (Cui et al., 2009).

Close to harvest time in both seasons (27/2/2010 and 24/2/2011), 24 fruit samples from the first three blocks ($n = 3$) were used for $\Delta^{13}C$ measurement. Samples were selected from the Nonpareil trees. In the laboratory, shells were separated from the other parts and were dried at a temperature of $61^{\circ}C$ for 3 days. After grinding and weighing into 3-4 mg capsules, the isotope composition (δ) was measured by using an isotope ratio mass spectrometer. The carbon isotope discrimination $\Delta^{13}C$ was calculated as the following equation (Hubick et al., 1986):

$$\Delta^{13}C = (\delta_a - \delta_p) / (1 + \delta_p) \quad \text{Equation 2.2}$$

δ_a = carbon isotope composition in atmosphere (-7.6×10^{-3})

δ_p = carbon isotope composition in plant tissue

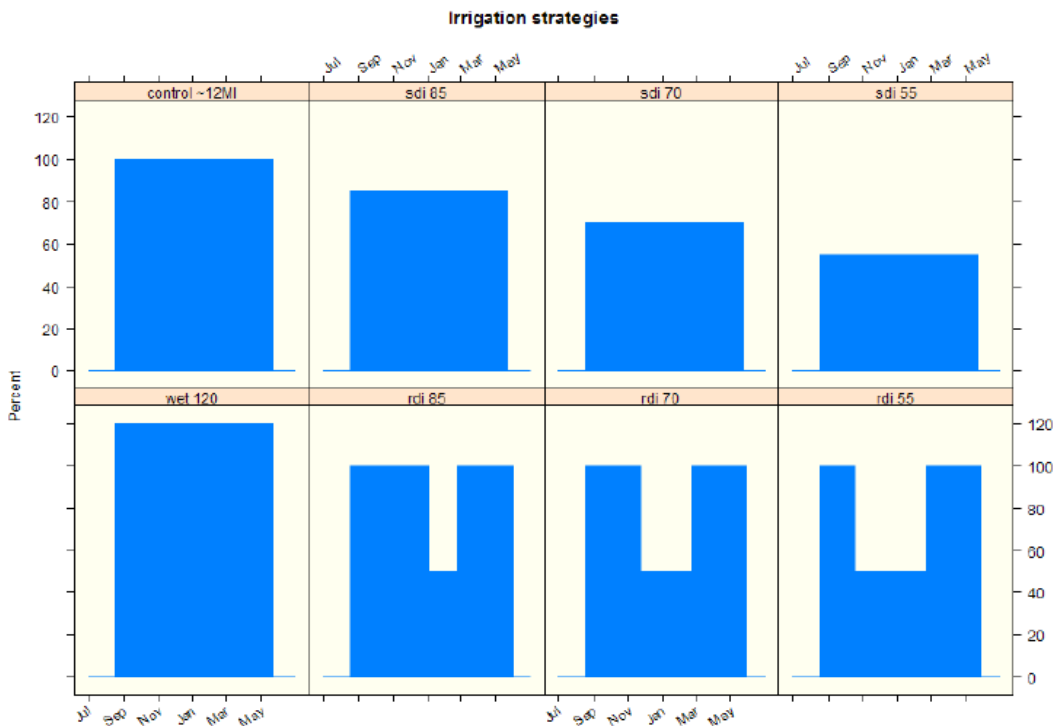
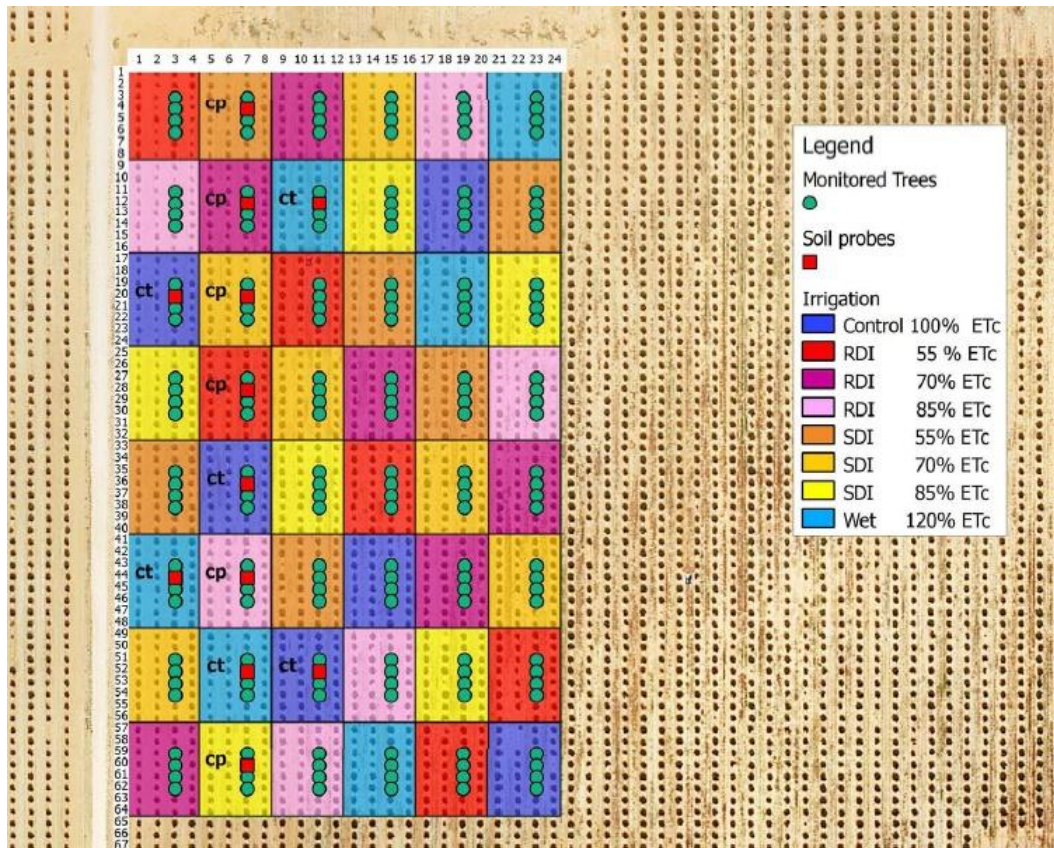


Figure 2.2 Two irrigation strategies and five watering levels. Y-axis depicts the percentage full ET_c . Flowering and fruit setting times were August and September respectively with harvest in March.

2.2.4 Statistical analysis

The trial was a randomised complete block design, including six blocks and eight treatment plots. Individual trees were monitored as indicated in Figure 2.1. However, $\Delta^{13}\text{C}$ and MSWP measurements were only applied for the first 3 blocks. Statistical analyses were applied using the analysis of variance (ANOVA). Means were separated by Duncan's multiple range tests at 5% level of probability. Analysis of variance was computed by Statistical Analysis Software (SAS Institute Inc. 2004, SAS/STAT, 9.1). For multiple measurements from a single replicate, one mean per replicate was calculated; then the seasonal mean was calculated from those 6 results. Differences between the slopes of linear regressions and second-order polynomial regressions were tested using extra sum-of-squares F test (GraphPad Prism 5).

2.3 Results

The totals of irrigation plus effective rainfall per season for control treatments were approximately 1121 mm and 1011 mm for first and second seasons, respectively (Table 2.2). In 2010-2011, ET_0 increased by 32% compared to the previous season, while the effective rainfall was 16% higher than in the first season. In fact, the evaporative demand in 2010-2011 was declined due to more humid and frequently overcast weather. Because of the lower evaporative demand in the second season, the volumes of water applied for all treatments were considerably lower in comparison with 2009-2010 season. However, deficit irrigation treatments failed to impact any considerable water stress on trees and, consequently, prevented any yield loss compared to well-watered treatments (Table 2.3). Table 2.2 gives a summary of irrigation volumes, effective rainfall and the timing of irrigation treatments applied during both 2009-2010 and 2010-2011 seasons.

Table 2.2 Irrigation treatments, irrigation, effective rain, effective rain + irrigation, reference crop evapotranspiration (ET_o) and timing of deficit in 2009-2010 and 2010-2011 seasons.

Season	Treatment	Irrigation (mm)	Effective rain (mm)	Irrig. + eff. rain (mm)	ET _o (mm)	Deficit Timing
2009-2010	1 con.	937	184	1121	1435	-
	2 wet	1131	184	1315		-
	3 sdi.85	806	184	990		all season
	4 sdi.70	694	184	878		all season
	5 sdi.55	534	184	719		all season
	6 rdi.85	836	184	1020		10/01/10- 17/02/10
	7 rdi.70	664	184	848		12/11/09- 17/02/10
	8 rdi.55	552	184	736		10/09/09- 17/02/10
2010-2011	1 con.	781	214	1011	1089	-
	2 wet	933	214	1170		-
	3 sdi.85	677	214	906		all season
	4 sdi.70	578	214	807		all season
	5 sdi.55	476	214	706		all season
	6 rdi.85	668	214	900		10/01/10 - 2/03/11
	7 rdi.70	508	214	739		12/11/09 - 2/03/11
	8 rdi.55	488	214	719		10/09/09 - 2/03/11

2.3.1 Kernel yield and WUE

Significant differences in kernel yield were found among irrigation treatments in the first but not second season (Figure 2.3A and Table 2.3). In 2009-2010 season, there were significant ($P < 0.05$) differences in the kernel yield between control and water deficit treatments irrigated at 70% ET_c or less. In other words, applying 85% ET_c did not significantly reduce the kernel yield. However, WUE (kernel yield/total water applied, kg/mm) was not significantly different among 70%, 85% and control treatments (Figure 2.3C).

Following the previous work by Goldhamer et al. 2006, deficit irrigations at 55%, 70% and 85% ET_c are respectively regarded as severe, moderate and mild treatments applied (Goldhamer et al., 2006). The severe treatments (SDI, RDI 55%) showed a significant ($P < 0.05$) reduction in kernel yield (about 17%) but significant increase in WUE (up to 27%) in regard to control treatments. Kernel yield was not significantly increased by applying extra water in the wet 120% ET_c treatments thus clarifying the lower values of WUE observed in this treatment (Figures 2.4A and C). Between different treatments, SDI 55%, RDI 55% and

RDI 70% represented more decrease in kernel fraction compared to control and wet 120% ET_c (Figure 2.3B).

In contrast with the first season, no significant differences in kernel yield and kernel fraction were observed during 2010-2011 season (Table 2.3). However, kernel yield in the second season was significantly (22%) reduced in comparison with 2009-2010 season. Meanwhile, because of cold weather in 2010-2011, there was 3 weeks delay in this season.

2.3.2 Plant water relations

2.3.2.1 Midday stem water potential (MSWP) and stomatal conductance (g_s)

In the first year, the seasonal MSWP values for control 100% ET_c trees progressively decreased over 4 months (from mid-October to mid-February), ranging between -0.58 and -1.14 MPa (Figure 2.5B). Eventually, both deficit strategies at 55% ET_c showed the greatest reduction in MSWP relative to control trees reaching values of below -2.6 and -3.0 MPa for SDI and RDI respectively. The same as kernel yield and fraction, the seasonal averages of MSWP significantly declined in SDI and RDI 55% and RDI 70% in comparison with control, wet 120% ET_c , RDI and SDI 85% ET_c (Figure 2.3D). Because of different rainfall patterns between the two consecutive seasons, the average of MSWP in the first season was significantly more negative (nearly double) than in the subsequent season (-1.04 and -0.54 MPa for 2009-2010 and 2010-2011, respectively). However, in the second season there were not significant differences in MSWP between different treatments (Table 2.3).

In the 2009-2010 season, the most significant differences in the seasonal average of g_s , measured under sunny conditions, were observed between well watered treatments and SDI 55%, RDI 55% and 70% ET_c treatments (Figure 2.3E). RDI 70% ET_c showed relatively lower g_s in comparison with SDI% ET_c ; however in 2009-2010 there were not significant differences between SDI and RDI treatment with the same levels of water applied. In the first

season, the time course of the g_s response to irrigation deprivation and its recovery after water resumption was approximately similar to the MSWP response, but there were more variations in the g_s of well watered treatments compared to MSWP. From late November there was a steady decrease in both MSWP and g_s until early February. By early March, MSWP and g_s were recovered to the same levels as well watered trees (Figure 2.5A).

2.3.2.2 Increment of trunk circumference (ΔTC)

Trunk circumference growth from the beginning to the end of the season, indicating trees growth rates (Grattan et al., 2006), was affected by irrigation treatments in 2009-2010 (Figure 2.3F). ΔTC measurement at the end of this season showed smaller increase for all deficit irrigations compared to control. The lowest values of ΔTC were observed in SDI 55%, RDI 55% treatments. The significant differences were observed between different water levels but not strategies. Despite the kernel yields, reducing irrigation from 85% to 70% did not significantly decrease trunk circumference growth in RDI 70%.

2.3.2.3 Carbon isotope discrimination

Carbon isotope discrimination values changed significantly between treatments in the first season. The 2009-2010 season revealed different trends with a tendency towards greater values of $\Delta^{13}C$ in control, wet, RDI 85% ET_c and SDI 85% ET_c compared to other treatments (Figure 2.3G). $\Delta^{13}C$ values in SDI and RDI 55% and RDI 70% treatments represented most differences with control and wet 120% ET_c . Nevertheless, in the second season, with inadequate water deficit treatments due to persistent humid weather and infrequent rainfall, no significant differences were observed between treatments. However, the average mean of $\Delta^{13}C$ in 2010-2011 season was significantly higher compared with the preceding season (Table 2.3).

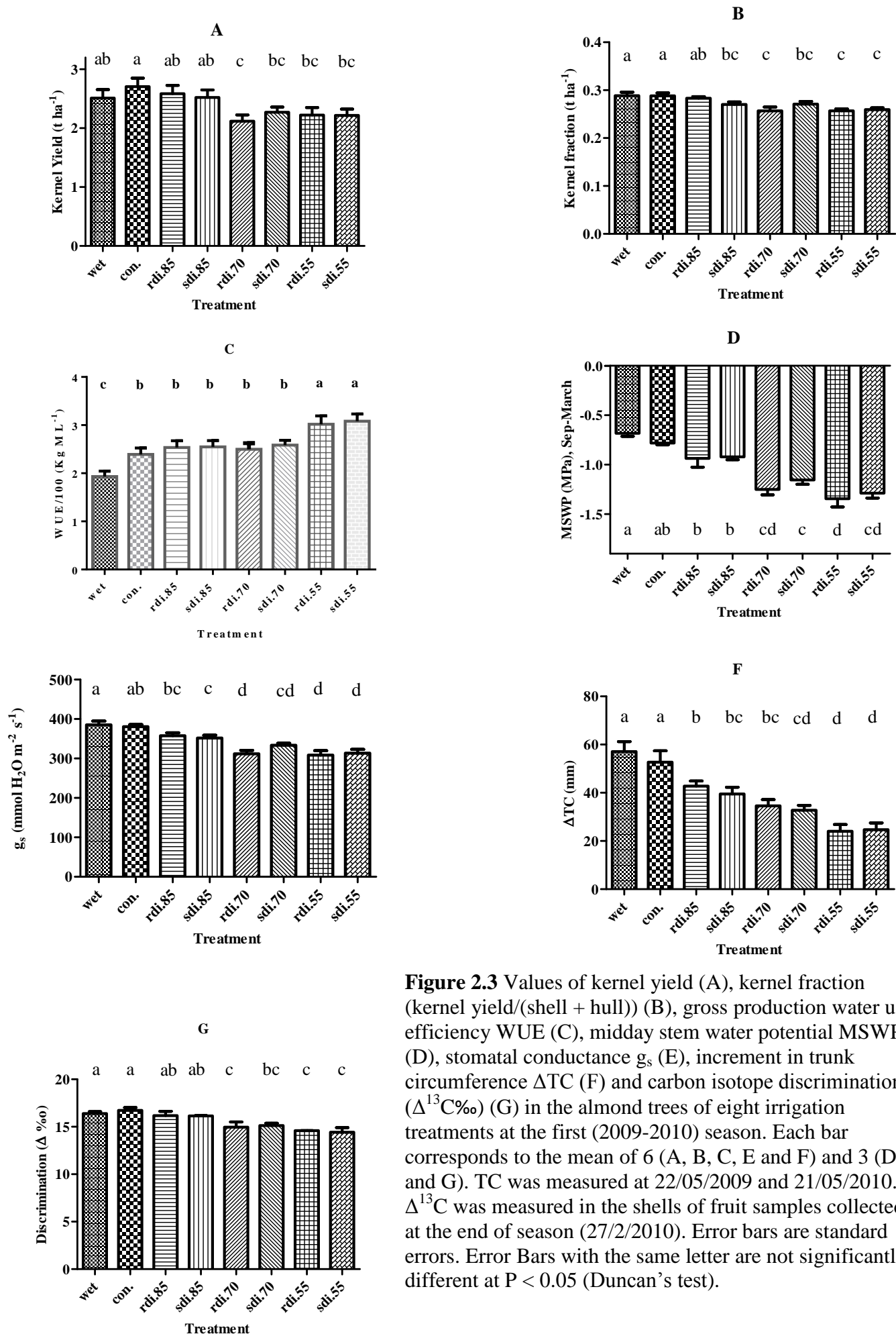


Figure 2.3 Values of kernel yield (A), kernel fraction (kernel yield/(shell + hull)) (B), gross production water use efficiency WUE (C), midday stem water potential MSWP (D), stomatal conductance g_s (E), increment in trunk circumference ΔTC (F) and carbon isotope discrimination ($\Delta^{13}C\%$) (G) in the almond trees of eight irrigation treatments at the first (2009-2010) season. Each bar corresponds to the mean of 6 (A, B, C, E and F) and 3 (D and G). TC was measured at 22/05/2009 and 21/05/2010. $\Delta^{13}C$ was measured in the shells of fruit samples collected at the end of season (27/2/2010). Error bars are standard errors. Error Bars with the same letter are not significantly different at $P < 0.05$ (Duncan's test).

Table 2.3 Variations of kernel yield (KY) ($\text{t}\cdot\text{ha}^{-1}$), kernel fraction (KF), carbon isotope discrimination $\Delta^{13}\text{C}\text{‰}$ and midday stem water potential (MSWP) (MPa). The factors are compared between eight different irrigation treatments in almond trees in (2010-2011) season. Values are means of 3 (MSWP and $\Delta^{13}\text{C}$) and 6 (KY and KF). Mean values within a column followed by different letters were significantly different at $P < 0.05$ using Duncan's comparison test. MSWP was measured from 29/09/2010 to 10/03/2011. The shells of the fruit samples collected at the end of season (24/2/2011) were used for $\Delta^{13}\text{C}$ measurement

Treatment	KY ($\text{t}\cdot\text{ha}^{-1}$)	KF (kernel/fruit)	MSWP (MPa)	$\Delta^{13}\text{C}\text{‰}$
wet	2.14 ^a	0.267 ^a	-0.523 ^a	20.02 ^a
con.	2.18 ^a	0.269 ^a	-0.513 ^a	19.90 ^a
rdi.85	2.14 ^a	0.261 ^a	-0.513 ^a	18.36 ^a
sdi.85	2.24 ^a	0.264 ^a	-0.52 ^a	16.99 ^a
rdi.70	2.11 ^a	0.258 ^a	-0.599 ^a	16.01 ^a
sdi.70	2.08 ^a	0.261 ^a	-0.519 ^a	17.11 ^a
rdi.55	2.08 ^a	0.255 ^a	-0.581 ^a	18.92 ^a
sdi.55	2.08 ^a	0.260 ^a	-0.53 ^a	17.27 ^a

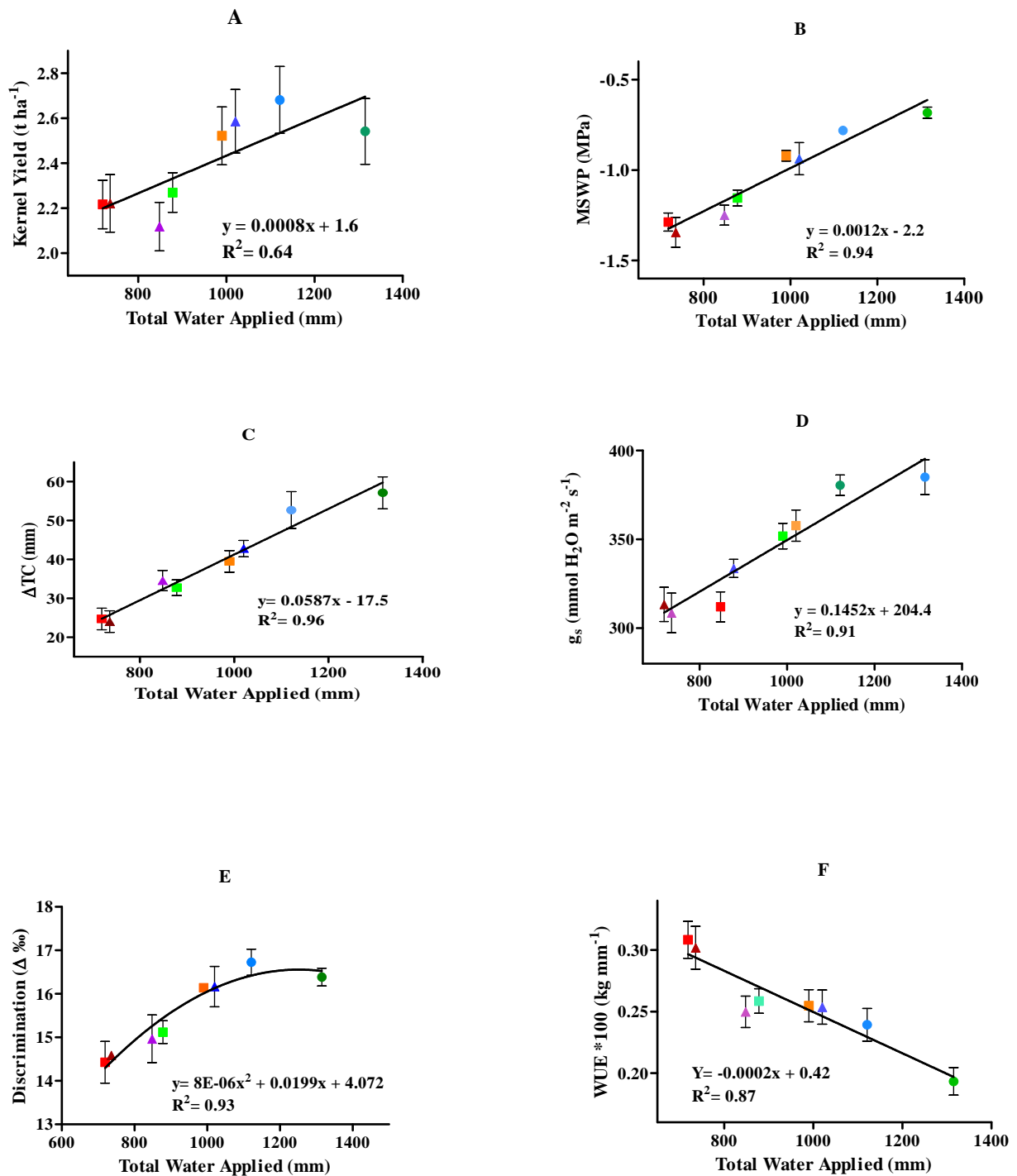


Figure 2.4 Relationship between the total amount of applied water, including rainfall, (mm/season) and kernel yield (A), midday stem water potential MSWP (B), trunk circumference ΔTC (C), stomatal conductance g_s (D), carbon isotope discrimination $\Delta^{13}C\text{‰}$ (E) and gross production water use efficiency WUE (F) for each deficit irrigation treatment with respect to control 100% ET_c over 2009-2010 season. Each point represents the mean of 6 (A, C, D, E and F) and 3 (B and E) replications \pm SE.

▲	rdi.55
▲	rdi.70
▲	rdi.85
■	sdi.55
■	sdi.70
■	sdi.85
●	con.
●	wet

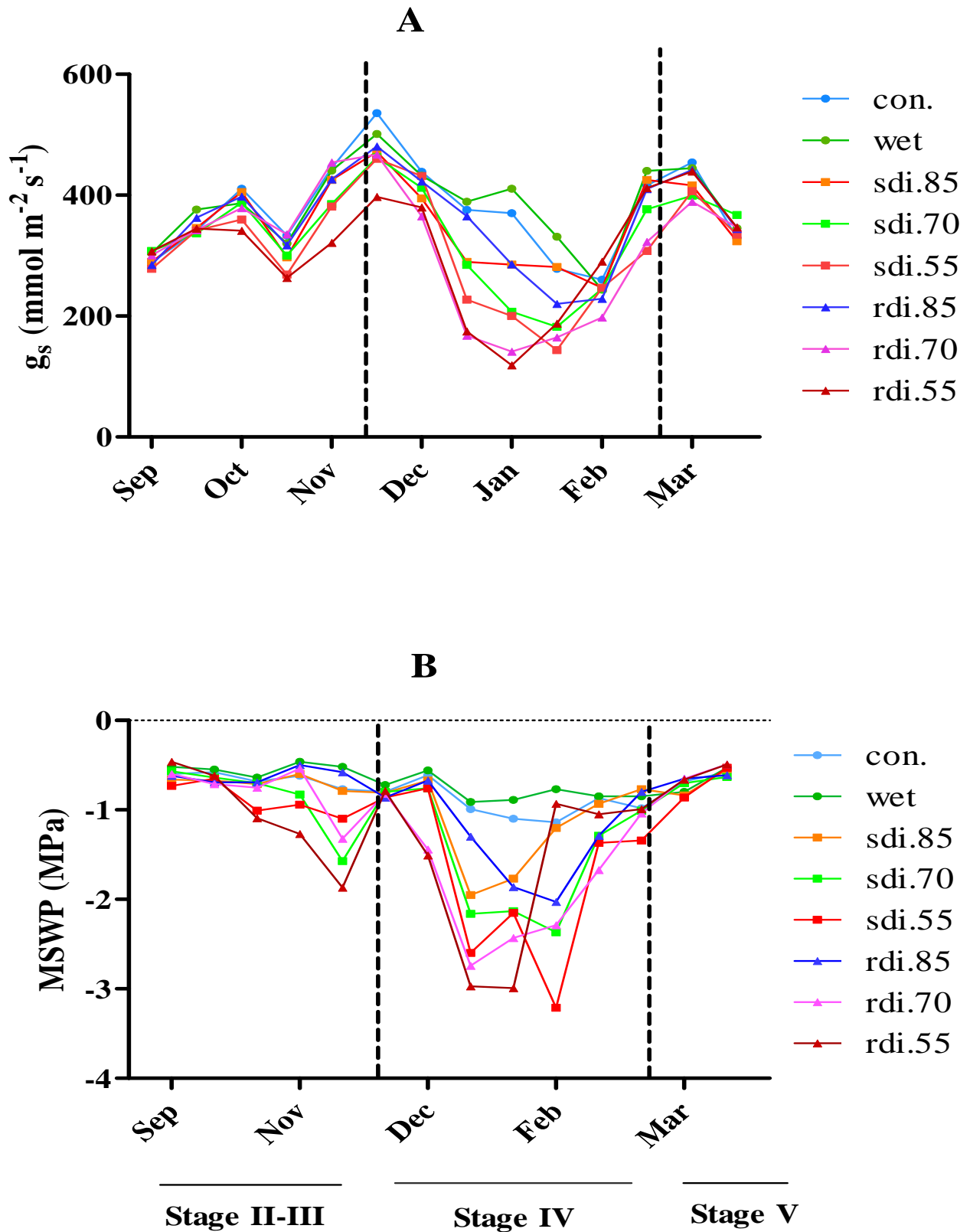


Figure 2.5 seasonal fluctuations in (A) midday stomatal conductance (g_s) and (B) midday stem water potential (MSWP) of almond trees grown in control and water-stressed conditions. The developmental stages (II-III: rapid vegetative growth, IV: kernel-filling, V: post-harvest) of *P. dulcis* tree are separated by dashed vertical lines.

2.4 Discussion

2.4.1 Water status indicators

Similar to the results obtained in this experiment (Figure 2.3A), other studies on almond trees also showed the deleterious effects of drought stress on either kernel size or kernel yield (Goldhamer and Viveros, 2000, Egea et al., 2010, Girona et al., 1993). However, Goldhamer et al. (2006) reported that there was no significant yield loss in 70% and 85% ET_c with respect to control treatments (Goldhamer et al., 2006). Romero et al. applied a severe (20% ET_c) RDI water deficit irrigation during kernel filling phase in almond trees before subsequent water resumption. They observed yield reduction only when water potential was below -2.0 MPa (Romero et al., 2004c). The results of this study show that for 70% ET_c of irrigation or less, stem water potential was below -2.0 MPa during the kernel filling period (Figure 2.5B). Related studies showed that such values of MSWP are an indicator of moderate to severe water deficit which may reduce productivity in almond trees (Shackel, 2007). Nevertheless, the varieties of almonds in this study were Nonpareil and Carmel, whereas Romero et al. selected Cartagenera for their experiment; hence, the conditions of the two experiments cannot be exactly the same, because different varieties may represent different thresholds of drought tolerance.

There were no significant differences in kernel yield between SDI and RDI when treated with the same water volumes over the season (Figure 2.3A). Accordingly, Goldhamer et al. (2006) observed no significant differences between the kernel yield of almond trees irrigated with the same volumes of water under SDI and pre harvest deficit irrigation strategies (Goldhamer et al., 2006). However, Fereres and Soriano (2007) observed in peach trees that for the same levels of irrigation, RDI was more productive compared to SDI (Fereres and Auxiliadora Soriano, 2007). By contrast, the results obtained in this study indicate that kernel yield in RDI 70% ET_c treatment was even slightly lower than SDI 70% ET_c . That is probably because of the severe water stress ($MSWP < -2$ MPa) during the kernel filling period in RDI 70% ET_c .

In fact, during kernel filling period the amount of water applied for RDI 70% ET_c treatment was 50% of control, whereas in SDI 70% it was 70% (Table 2.1). However, the volumes of water applied during kernel filling period for both RDI and SDI 55% were not remarkably different (50% and 55% of control). It is presumably the reason for the non significant differences in kernel yields between RDI 55% and SDI 55% treatments. Goldhamer and Viveros (2000) observed that applying severe water deficit treatments during kernel filling period may slightly reduce kernel yield in almond trees, whereas mild drought treatment has no negative effect on kernel yield (Goldhamer and Viveros, 2000). Although in kernel filling stage the sensitivity of almond orchards to water stress is lower compared to other stages, severe water deficit during this period can decrease tree leaf area, which consequently reduces kernel yield (Romero et al., 2004c) (Figures 2.4A). Meanwhile, related studies showed a greater sensitivity of kernel growth in almond orchards under pre-harvest RDI in comparison with SDI (Goldhamer et al., 2006).

Applying more water at wet 120% ET_c compared to control 100% ET_c did not increase kernel production (Figure 2.3A). Similarly, in four consecutive years of study by Girona et al. (2005), the optimal yield in almond trees was obtained at full ET_c (100% ET_c) irrigated treatments, instead of excess water treated trees (Girona et al., 2005). In spite of some reports that the intensity of water deficit has no effect on the kernel fraction (kernel-to-fruit ratio) (Egea et al., 2010, Romero et al., 2004a, Torrecillas et al., 1989), in this study significant differences were observed between well watered and water deficit treatments in the first year (Figure 2.3B). Most of the biomass of hulls and shells grows early in the seasons when stresses are less severe. Kernels on the other hand fill much later and therefore are probably more susceptible to stress that is more severe in December than earlier in the season. According to previous studies on almond trees more than 50% of pericarp nutrition content transfers to the kernel during kernel filling period (Weinbaum and Muraoka, 1986). Meanwhile, MSWP values for SDI 55%, RDI 55% and RDI 70% treatments in kernel filling period changed between -2.29 and -3.21 MPa which is regarded as moderate to severe water

deficit for almond trees (Shackel et al., 1998). Therefore, the significant decrease of kernel fraction (kernel-to-fruit ratio) in SDI 55%, RDI 55% and RDI 70% compared to well watered treatments in 2009-2010 season (Figure 2.3B) is probably due to the deleterious effects of severe water deficit on nutrition remobilization from pericarp to kernels.

The steady decrease of MSWP values for control treatments in the first season was presumably a result of the increased evaporative demand during the season and the inability of the plant's hydraulic system to supply a flow of water at a rate to match maximum transpiration from the canopy (Ortuno et al., 2009, Shackel et al., 1997, Shackel, 2007). For RDI treatments, MSWP was similar to those recorded in control trees, except during the periods with irrigation deficits. Due to the sudden increase of vapour pressure deficit (VPD) and temperature in late November, MSWP was dramatically reduced especially in water deficit treatment (Figure 2.5B). Meanwhile, there was a recovery in MSWP from early February in deficit irrigation treatments (Figure 2.5B). That is probably because of the recovery of soil water content or the relative decrease in VPD and temperature in this period. The other reason can be the absence of strong fruit sink activity (Romero and Botía, 2006) towards the later stages of kernel filling which decreases the demand for water after early February.

In 2009-2010 season, compared with the MSWP of well watered treatments, g_s values represented more variations between different measuring dates, presumably in response to predominant environmental conditions (Klein et al., 2001). Besides water stress, there are other environmental factors which can affect stomatal behaviour, such as temperature, light, air humidity, etc (Yu et al., 2004, Arve et al., 2011, Hiroyuki et al., 2010). In this experiment, g_s measurements were taken only in sunny days and not cloudy conditions; otherwise the level of g_s between different measuring dates was more variable than the current results. The results of this experiment also confirm the related studies by Wartinger et al. (1990) and Egea et al. (2011) that almond trees follow the anisohydric pattern (Wartinger et al., 1990, Egea et al., 2011). In anisohydric behaviour, stomata are less responsive to water deficit and therefore,

MSWP declined more than in isohydric plants (Chaves et al., 2010, Tardieu and Simonneau, 1998). The results obtained in this study show that for both RDI and SDI 55% treatments, the average of MSWP values during the first season were decreased by 39% compared to control, whereas g_s values were reduced only by 18% (Figure 2.3D and E). Since, MSWP and g_s values for RDI treatments change drastically in short periods of the season (when deficit irrigations were applied); therefore the averages of the whole season were used to compare g_s and MSWP variations between RDI (short-term treatment) and SDI (long-term treatment). In RDI treatments, although the water deficit was applied for a short period but it was relatively more severe compared to SDI treatments. Therefore, RDI treatments showed lower g_s than SDI treatments with the same levels of irrigation (Figure 2.5A). Meanwhile, after rewatering there was a relative delay in the stomatal reopening of those trees treated with 70% RDI in comparison with 70% SDI treatment. Such slow reopening of stomata after severe water deficit in plants might be useful for turgor recovery (Mellisho et al., 2011, Torrecillas et al., 1999, Brodribb and Cochard, 2009, Resco et al., 2009).

In the first season, the greatest difference for ΔTC was observed between both RDI and SDI 55% ET_c with control 100% and wet 120% ET_c treatments (Figure 2.3F). As reported before in almond and olive trees (Grattan et al., 2006, Romero et al., 2004b), the largest growth rate of trunk circumference was observed with the highest amount of water application. Trunk growth of control trees was 54% greater than that of SDI and RDI 55% ET_c treatments. Results obtained in this experiment showed that below the threshold level of 1121 mm of irrigated water, the seasonal ΔTC significantly declined. Also there were significant reductions in ΔTC when the seasonal amounts of applied water were reduced from 1005 to 863 mm or from 863 to 728 mm (Figure 2.4C). Trees irrigated with the same levels of water showed nearly similar percentages of trunk growth relative to total circumference. This result indicates that trunk girth expansion is highly positively correlated with the seasonal volume of applied irrigation as reported before in almond trees (Figure 2.4C) (Egea et al., 2010, Girona et al., 1993, Hutmacher et al., 1993, Romero et al., 2004b). In other words, the sensitivity of

trunk growth to water deficit is not dependent on timing, but is more affected by the severity of water stress. The influences of the timings of severe water deficit on ΔTC are relatively different to impacts on kernel fraction (Figure 2.3 B); probably because during maximum evaporative demand, which is at late summer, the process of vegetative growth is very much reduced in almond trees. The trunk growth period for almond trees occurs mostly in spring, and continues more slowly during kernel filling (Romero et al., 2004a). For kernel yield and kernel fraction on the other hand, the period of kernel filling for the majority of almond varieties occurs during the late summer (Girona, 1992, Kester et al., 1996). Although, there were no significant differences between ΔTC of different strategies with the same volumes of water, SDI 70% and SDI 85% ET_c exhibited slightly lower increases in trunk diameter compared to RDI treatments which were irrigated by the same levels of water (Figure 2.3F); presumably because deficit irrigation for SDI treatments was applied during the whole season, including rapid growth periods, while for RDI 70% and 85% ET_c water deficit was limited to the late summer when growth rate was not as high as the rapid growth periods.

There was a relatively close correlation between kernel yield and ΔTC in 2009-2010 (Figure 2.8B). In the same way, Romero et al. (2004) observed a significant correlation between kernel yield and ΔTC in almond trees (Romero et al., 2004b). In agreement with the results of Girona et al., 2005, no significant extra growth of trunk circumference was observed in wet 120% ET_c over control 100% ET_c treatments (Figure 2.3F) (Girona et al., 2005). However, some related studies on almonds implied the enhancement of vegetative growth in response to applying more water in addition to full irrigation (control 100% ET_c) (Shackel et al., 1998, Hutmacher et al., 1993).

The second order polynomial relationship ($P < 0.001$) between $\Delta^{13}C$ and applied watering volumes in 2009-2010 season (Figure 2.4E) was in agreement with several related studies (Gaudillere et al., 2002, Johnson et al., 1990, Kumar and Singh, 2009). Similar to other water status indicators in this experiment, $\Delta^{13}C$ values also were affected significantly by the

amounts of applied water rather than irrigating strategies. There was however a modest difference between RDI 70% ET_c and SDI 70% ET_c .

The weak overall correlations (data not shown) between kernel yield, water status indicators and water applied for the second season does not mean that the parameters are unrelated; it is most probably due to the humid weather and frequent rain (Table 2.2) that negated the possible effects of deficit irrigation on almond trees. In this regard, MSWP and $\Delta^{13}C$ measurements for the second season (Table 2.3) indicated that almond orchards experienced totally weak and infrequent water stress in 2010-2011 season.

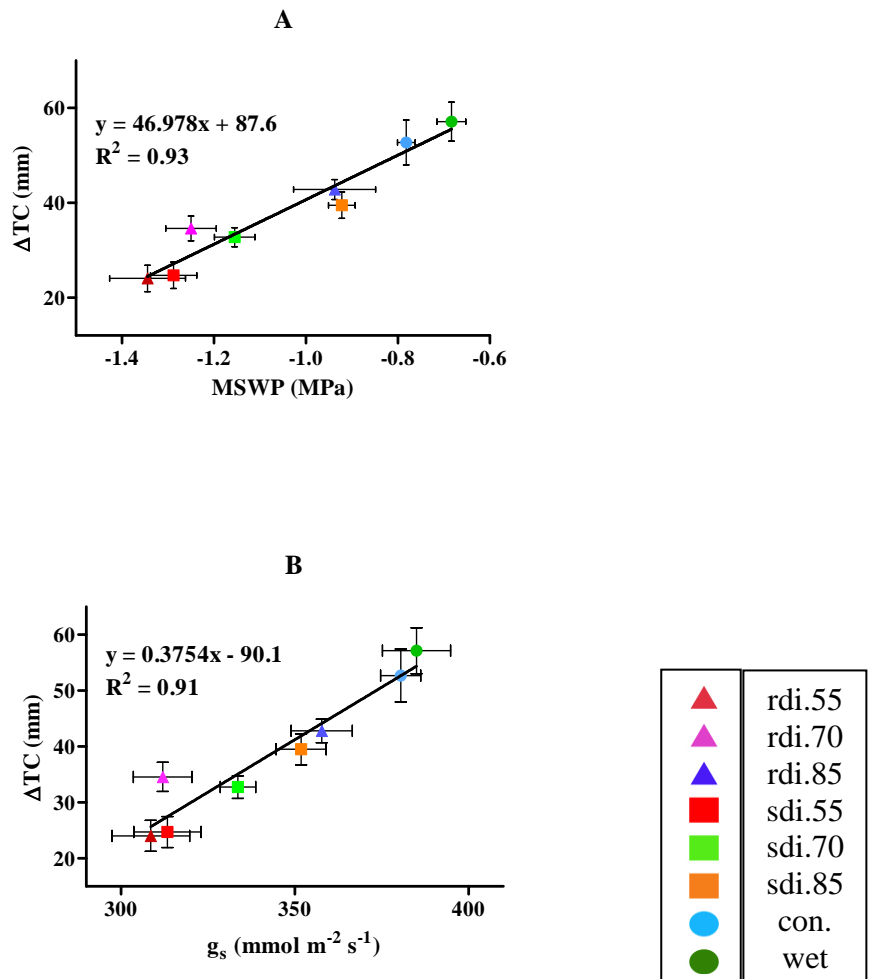


Figure 2.6 Relationship between trunk circumference variations (ΔTC) with the average midday stem water potential (MSWP) (A) and stomatal conductance (g_s) (B). Data are recorded from the almond trees irrigated with eight different regimes in 2009-2010 season. Each point is the mean of 6 (g_s and ΔTC) and 3 (MSWP) replicates $\pm SE$, ($P < 0.001$).

2.4.2 Relationships between water status indicators

In 2009-2010 season, during which a range of irrigation deficits was applied, highly significant correlations ($P < 0.01$) were observed among plant water status indicators and the amount of water applied. These relationships indicate that such parameters may be useful for estimation of drought severity (Figures 2.5A, B, C, D, E and F). The evident responses of MSWP to a range of water deficits (Figure 2.4B) are comparable with previous results in almond (Nortes et al., 2005), plum (Intrigliolo and Castel, 2006a) and apple trees (Doltra et al., 2007). Therefore, MSWP is a good indicator of water stress ($R^2 = 0.94$, $P < 0.01$), especially for site specific irrigation scheduling (Shackel et al., 1997). Corresponding with related studies, the variations in vegetative growth were closely related to stem water potential ($R^2 = 0.93$, $P < 0.01$, Figure 2.6A) (Shackel et al., 1997, Romero et al., 2004b). This behaviour is important for the adaptation of plants to stressful conditions (Shackel et al., 1997).

The deleterious effects of water deficit on growth are reported by several studies (Kriedemann et al., 1981, Berman and DeJong, 1997, Ortuno et al., 2004). Presumably due to water deficit a reduction occurs in carbon assimilation and thus growth can be limited (Chaves et al., 2009, Muller et al., 2011). Moreover, according to laboratory experiments water deficit may reduce cell turgor (Kirnak et al., 2001, Dale, 1988) which subsequently limits turgor-dependent processes including both cell elongation and g_s ($P < 0.01$, Figure 2.4D). The consequence is the reduction of photosynthesis and leaf expansion that reduces vegetative growth ($P < 0.01$, Figure 2.6B) (Berman and DeJong, 1997, Aguirrezabal et al., 2006). In addition, water deficit may shift the allocation pattern of photosynthetic materials to different parts of the plant (Kramer, 1983, Schulze, 1986b). Meanwhile, under severe water deficit conditions, metabolic limitations and the disruption of enzymatic reactions involved in photosynthesis may be an additional reason for suppressing the growth (Baldocchi et al., 1985, Flexas et al., 2006, Flexas et al., 2004, Schulze, 1986a). Diminished tree size and less fruiting positions are the consequences of limited growth due to water deficiency (Esparza et al., 2001). Similar to the

results of this experiment, Romero et al (2004) also observed a correlation between ΔTC , as a growth parameter, and kernel yield in almonds (Figure 2.8B) (Romero et al., 2004b).

Fluctuations in g_s , particularly in water deficit treatments, throughout the season were correlated with those in stem water potential (Figures 2.6A, B); hence in January, with maximum rates of evapotranspiration, both MSWP and g_s were at the minimum levels. The close correlation ($P < 0.001$) between g_s and MSWP (Figure 2.7) has been reported in previous studies (Egea et al., 2011, Castel and Fereres, 1982, Garcia-Orellana et al., 2007).

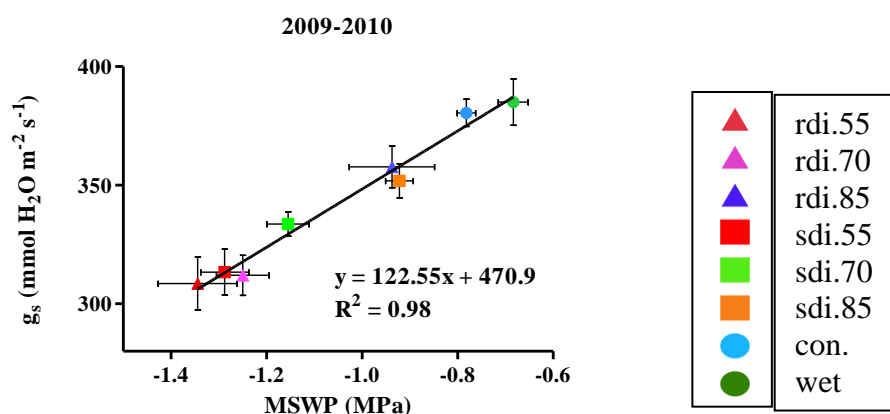


Figure 2.7 Relationship between midday stem water potential (MSWP) and stomatal conductance (g_s) of almond trees over the same season under eight irrigation regimes. Each point is the mean of 6 (g_s) and 3 (MSWP) replicates \pm SE.

Although some studies suggested MSWP and g_s as reliable water deficit indicators in orchards (Naor, 2000, Ballester et al., 2013, McCutchan and Shackel, 1992), because of some limitations, MSWP and g_s cannot be considered as completely effectual plant-based techniques for irrigation scheduling. For instance, frequent MSWP and g_s measurements throughout the season are necessary for monitoring water status in plants for the whole season (Ortuno et al., 2009). Accordingly, high labour requirements limit the prevalence of using MSWP and g_s for commercial irrigation scheduling (Dzikiti et al., 2010, Fereres and Auxiliadora Soriano, 2007).

Several studies demonstrate that $\Delta^{13}\text{C}$ is an integrative indicator of cumulative water stress in plants (Bladon et al., 2007, Loustau and Porté, 2001, Van Leeuwen et al., 2009, Poss et al., 2000). The main benefit of this approach, compared with MSWP and g_s , is that the whole seasonal tree-stress experiences are biochemically documented in carbon fractionations, whereas MSWP and g_s only depict the plant water status at an individual time. In fact, MSWP and g_s cannot demonstrate the water deficit stresses that trees may have withstood before then. Meanwhile, the effects of short term stresses on MSWP and g_s will be underrepresented by averaging it out with the whole season. As a result, MSWP and g_s can be used as effective indicators for day-to-day irrigation scheduling, but $\Delta^{13}\text{C}$ is more suitable for the validation of watering schemes at the end of the season. In other words, $\Delta^{13}\text{C}$ may not be useful for irrigation scheduling during the growing season but can help to test different irrigation strategies at harvest (Doltra et al., 2007, Gaudillere et al., 2002, Van Leeuwen et al., 2010). In fact, using $\Delta^{13}\text{C}$ values provides an integrated record of seasonal plant water stress that can help growers to design more precise irrigation schedules compared to using MSWP and g_s (Grattan et al., 2006, Farquhar et al., 1989).

Measuring $\Delta^{13}\text{C}$ can be more useful when there is a considerable time gap between planting and the commencement of fruiting in the trees. In almond orchards, the fruit production begins after the third year of planting but the full bearing status can take 5-6 years (Griffiths and Huxlen, 1992). Therefore, applying the $\Delta^{13}\text{C}$ method for almond trees helps to compare the WUE between different almond genotypes at least 3 years before the beginning of fruit production. However, measuring $\Delta^{13}\text{C}$ at early growth stages cannot be useful for formulating a suitable irrigation schedule, because $\Delta^{13}\text{C}$ changes significantly as trees develop from an early vegetative structure to the kernel production stage (Poss et al., 2000).

Relationships between water applied and water status indexes (Figures 2.4D, E, F and G) indicated that ΔTC was the most sensitive indicator of water status in almond trees, followed by MSWP, g_s and $\Delta^{13}\text{C}$, respectively. The lower variability under various treatments confirmed g_s and $\Delta^{13}\text{C}$ as the least sensitive water stress indicators. Presumably, because of

the anisohydric behaviour of almond trees, g_s showed lower sensitivity to water deficit in comparison with MSWP (Wartinger et al., 1990, Egea et al., 2011). In this regard, $\Delta^{13}\text{C}$ variations also reflect the stomatal behaviour, and therefore represented lower sensitivity to water stress compared to other water status indicators (Farquhar and Sharkey, 1982, Heldt and Piechulla, 2011). However, previous complementary studies suggested that selecting a sensitive water status indicator for irrigation scheduling depends on the age of the trees, the possible number of records, water deficit level and the length of stress period (Doltra et al., 2007, Ortuno et al., 2010).

Between water status indicators, ΔTC showed lower correlation with kernel yield in comparison with MSWP, g_s and $\Delta^{13}\text{C}$ (Figures 2.9A, B, C and D); Therefore, MSWP, g_s and $\Delta^{13}\text{C}$ are more suitable rather than ΔTC for predicting yield in almond trees. Consistent with these results, Intrigliolo and Castel also reported the relative accuracy of MSWP and g_s for yield prediction compared to ΔTC in Plum trees (Intrigliolo and Castel, 2006a). Bearing in mind that direct measurement of yield, particularly with large numbers of replicates and lines, is a time consuming and costly process, $\Delta^{13}\text{C}$ assessment can be used as a rapid and convenient measure to predict yield (Figure 2.8D) (Hall et al., 1997, Anyia and Herzog, 2004, Farquhar and Richards, 1984). In agreement with previous reports (Seibt et al., 2008, McCarthy et al., 2011), the correlation between $\Delta^{13}\text{C}$ and WUE in 2009-2010 season (Figure 2.9B) was not very strong ($R^2 = 0.61$). It is probably because of the respiration and transpiration from non-photosynthetic tissues that can affect the total carbon gain or water loss during the season (Cernusak et al., 2007, Seibt et al., 2008).

Due to the high rainfall in 2010-2011, poor results were obtained when $\Delta^{13}\text{C}$ values were plotted with WUE for the same season (data not shown). The late season onset, owing to cold weather, possibly contributed to suboptimal weather during pollination. Therefore, there was not a successful pollination and nut set in the second season. Meanwhile, the frequent rain and humid climate in 2010-2011 resulted in hull rot infection that could lead to yield loss (Teviotdale and Michailides, 1995). On the other hand, trees experienced very mild and

infrequent water deficit condition during the second season (Table 2.3), therefore $\Delta^{13}\text{C}$ was not significantly different between treatments. In fact, yield reduction in 2010-2011 season was mostly affected by other parameters rather than water deficit, which could be the reason for the weak correlations between WUE and $\Delta^{13}\text{C}$ or other water status indicators.

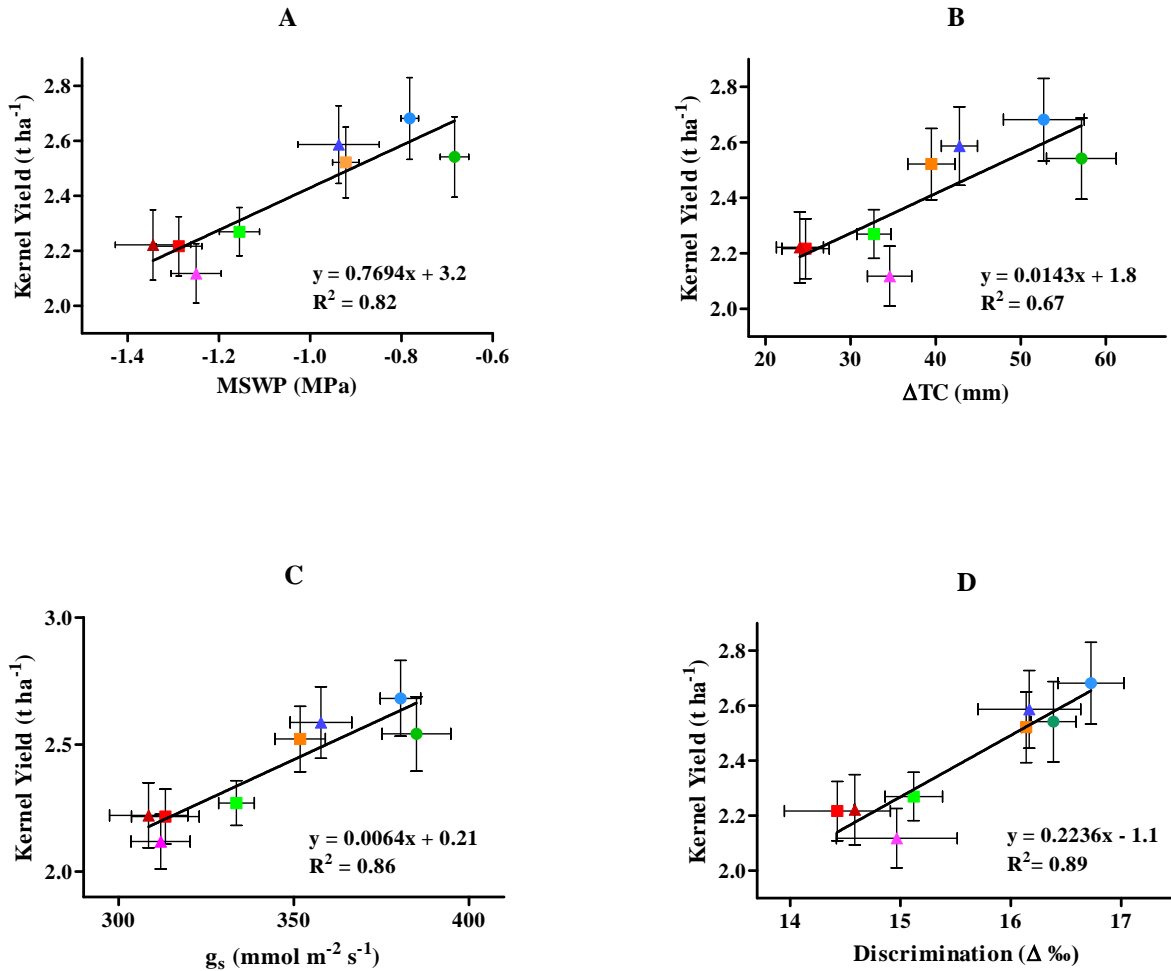


Figure 2.8 Relationship between kernel yield ($\text{t}\cdot\text{ha}^{-1}$) and water status indicators over 2009-2010 season. Each point represents the mean of 6 (kernel yield, ΔTC and g_s) and 3 (MSWP and $\Delta^{13}\text{C}$) replications \pm SE. ΔTC : trunk circumference. MSWP: midday stem water potential.

▲	rdi.55
▲	rdi.70
▲	rdi.85
■	sdi.55
■	sdi.70
■	sdi.85
●	con.
●	wet

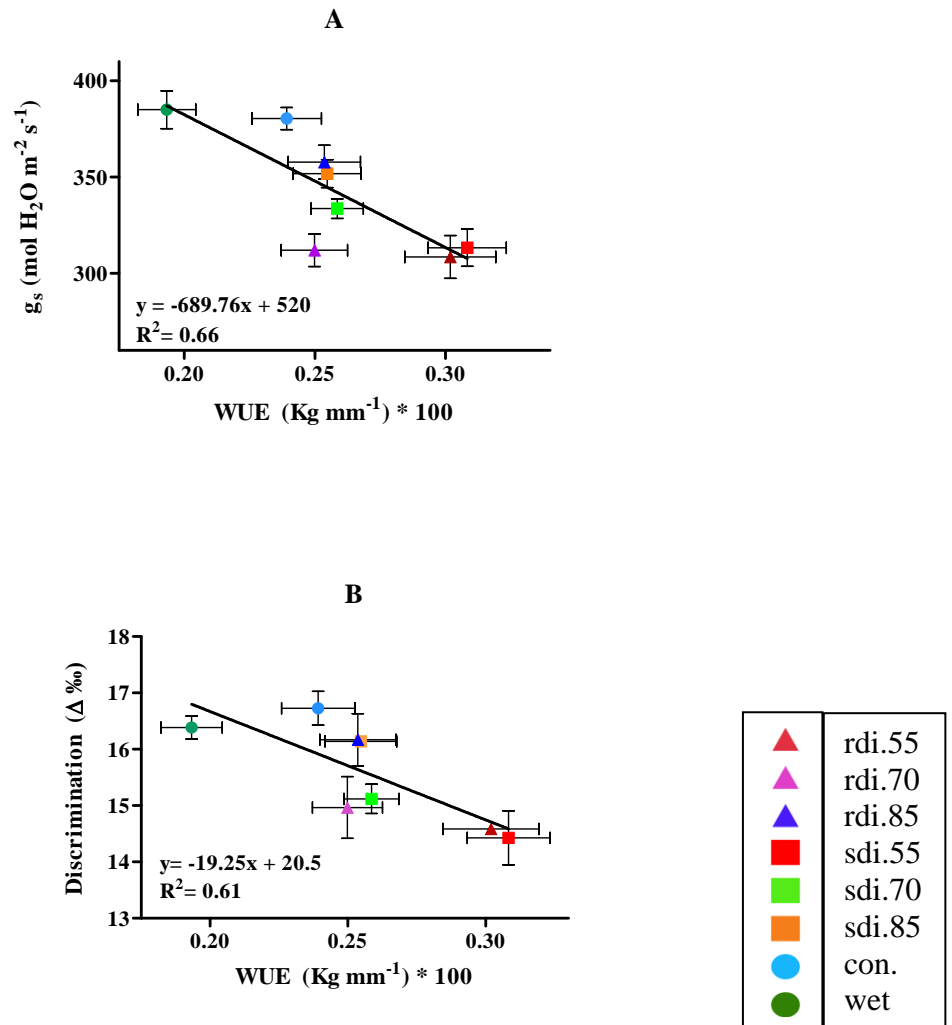


Figure 2.9 Relationship between gross production water use efficiency (WUE) * 100 with stomatal conductance (g_s) (A) and carbon isotope discrimination ($\Delta^{13}\text{C}\text{‰}$) (B) water status indicators over 2009-2010 season. Each point represents the mean of 6 (WUE and g_s) and 3 ($\Delta^{13}\text{C}$) replications \pm SE.

CHAPTER THREE

Water relations in almonds

3.1 Introduction

Improving WUE in perennial crops like fruit trees may decline water use without reducing yield. This can be particularly important for water limited areas in which crop productivity is dependent on water availability (Singh et al., 2009, Anyia and Herzog, 2004, Bassett et al., 2011). To this aim, it is necessary to identify the physiological processes involved in improving WUE in crops (Boyer, 1982, Raiabi et al., 2009).

Plant scientists use instantaneous water use efficiency WUE_i (A/E) as a direct measure of leaf level water use efficiency at a moment in time (Comstock and Ehleringer, 1992, Ripullone et al., 2004). For measuring WUE_i , the instantaneous CO_2 absorption (assimilation A) is compared to the instantaneous transpiration (E) through the stomata. A and E can be influenced by two factors: first is stomatal conductance (g_s). The other factor is the concentration differences between outside and inside for CO_2 ($c_a - c_i$) and water vapour ($w_i - w_a$) (Equations 3.1 & 3.2). Therefore, A and WUE_i are mostly affected by the function of stomata (Equation 3.3) (Lambers et al., 2008, Condon et al., 2002).

$$A = g_c (c_a - c_i) \quad \text{Equation 3.1}$$

$$T = g_w (w_i - w_a) \quad \text{Equation 3.2}$$

$$WUE_i = A/T = [g_c (c_a - c_i)]/[g_w (w_i - w_a)] \quad \text{Equation 3.3}$$

Since stomatal behaviour follows the optimality theory for gas exchange regulations, therefore it is also possible that A affect the variations of g_s . According to this theory, assimilating the maximum levels of carbon per unit of water transpired is considered as the optimal control of gas exchange (Cowan, 1977, Cowan and Farquhar, 1977).

In addition to the stomatal limitation, internal or non-stomatal limitations may also affect A . Non-stomatal limitations can be related to biochemical factors, e.g. photosynthetic enzyme activities (Faver et al., 1996) or chlorophyll content (Guerfel et al., 2009), and diffusive limitations, including mesophyll conductance (g_m) (Ethier and Livingston, 2004, Grassi and Magnani, 2005). During the year, non-stomatal limitations are dependent more on diffusional rather than biochemical factors. Biochemical limitations on the other hand can be important only under severe water deficit conditions or during leaf development and senescence (Grassi and Magnani, 2005).

g_m is defined as the conductance of CO_2 from sub-stomatal cavities to the carboxylation sites located in chloroplast stroma (Ethier and Livingston, 2004). This Pathway involves with different resistances in two phases, including gaseous phase in leaf intercellular air spaces, and aqueous phase inside the mesophyll cells (Gillon and Yakir, 2000, Cano et al., 2013). Previous findings indicate that g_m is finite and variable, and thus can have a large effect on A (Niinemets et al., 2009, Ethier and Livingston, 2004). Nevertheless, the role of g_m in A limitation analysis is still not fully understood, as in some studies (Ellsworth, 2000, Wilson et al., 2000) g_m is not even considered as a factor involving in non-stomatal limitations. However, the impacts of leaf anatomical traits on g_m are reported by several studies (Tosens et al., 2012, Tholen and Zhu, 2011, Tomás et al., 2013). In fact, the anatomical differences in the distances between sub-stomatal pathways to carboxylation sites might be the reasons for the variations in A (Evans and Von Caemmerer, 1996). In this respect, Brodribb et al. 2007 reported the considerable effects of leaf anatomical parameters on both g_m and A , confirming the close link between water and CO_2 pathways in the mesophyll (Brodribb et al., 2007).

Several studies indicate that g_m and g_s are highly correlated with each other, although not under all occasions (Perez-Martin et al., 2009, Flexas et al., 2012); probably because water and CO_2 transfer through a shared pathway in some parts in leaves. Both water vapour and CO_2 cross the aerial sub-stomatal cavity through the stomata (Flexas et al., 2012). Moreover, although liquid water and CO_2 diffuse mostly in the opposite direction, they share diffusion pathways in some parts in the post-venous area of the mesophyll (Evans et al., 2009, Terashima et al., 2011). Accordingly, previous studies demonstrated the strong correlation between g_m and the water transport capacity (hydraulic conductance) of the leaf (k_{leaf}) for various plant species (Flexas et al., 2012). In this respect, Sack and Frole (2006) observed that maximum A is highly dependent on the capacity of leaf hydraulic system to supply water for mesophyll photosynthetic cells (Sack and Frole, 2006). Therefore, it can be concluded that k_{leaf} is highly correlated with photosynthetic capacity, and thereby indirectly affects g_s by limiting the A in mesophyll cells (Brodribb et al., 2007, Addington et al., 2004). According to previous reports, there is more resistance to water movement in living mesophyll cells compared to highly conductive vessels (Passioura, 1988). Sack et al (2003, 2006) reported that extra-vascular resistance in the leaves of dicotyledons constitutes approximately 30% of the hydraulic resistance for the whole plant (Sack et al., 2003, Sack and Holbrook, 2006); therefore, vascular delivery of water is more effective in comparison with water flowing through the mesophyll cells. Based on this concept, it can be concluded that the post-venous hydraulic distance (D_m) lengths is correlated with the photosynthetic capacity of the mesophyll tissues (Figure 3.1) (Brodribb et al., 2007). That is why the spatial arrangement of minor veins in leaves is an important non-stomatal limiting factor for photosynthesis (Ocheltree et al., 2012, Brodribb et al., 2007).

Besides anatomical parameters, k_{leaf} can also be influenced by the age and the stage of development of leaves. In this regard, mature leaves show higher k_{leaf} in comparison with both young and old (close to senescence) leaves (Aasamaa et al., 2005, Brodribb et al., 2005). Meanwhile, different environmental conditions, e.g. leaf water status, light, temperature and

plant growing conditions can also lead to k_{leaf} variations (Sack and Holbrook, 2006, Brodribb et al., 2005).

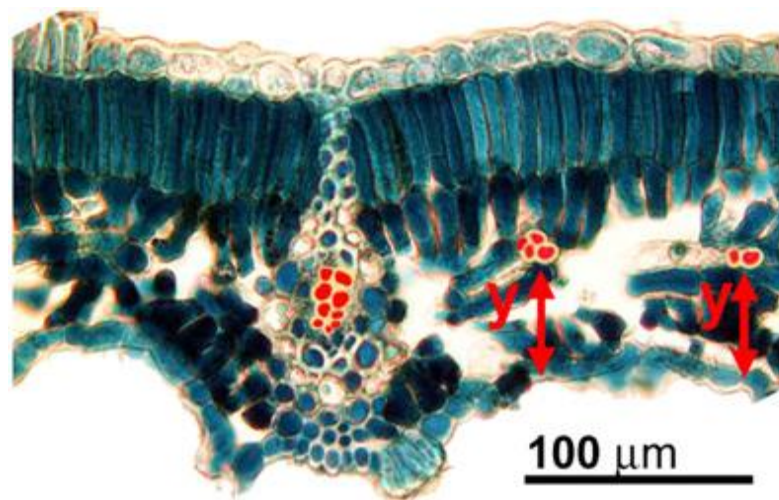


Figure 3.1 Cross sections from the leaves of *Curatela americana*. Red arrows depict the post venous distances. “y” letters indicate the vertical distance from vascular tissue to the leaf surface (Brodribb et al., 2007).

The main aim of this work was to compare the photosynthetic capacity and WUE_i of 5 almond breeding lines, mostly including the progenies of Nonpareil and Carmel, under non stress conditions. Meanwhile, the anatomical differences affecting g_m in the leaf tissues of Nanpareil, Carmel and Masbovera were examined. In comparison with measuring stomatal limitations, the estimation of g_m , as a non-stomatal factor is more complicated. Nevertheless, by using new methods and modern equipment the number of findings about g_m and anatomical issues in recent years has increased (Cano et al., 2013). In this study, images prepared by cryo-scanning electron microscopy (cryo-SEM) method were used to compare the D_m and the anatomy of mesophyll tissue in Nanpareil, Carmel and Masbovera.

3.2 Materials and Methods

3.2.1 Experiment 1

According to the characteristics of the parental lines, 5 mixed crosses of almond (Carmel x Tarraco, Johnston x Lauranne, Nonpareil x Tarraco, Nonpareil x Lauranne and Nonpareil x Vayro), were selected with 4 replicates for the experiment. Pots were arranged randomly in each block on 4 separated benches as replicates. Trees were planted at a same time in 2008. Each tree was grown in a 30 cm pot containing coco peat mix (2/3 peat, 1/3 sand) plus slow release fertiliser. Pots were maintained in a greenhouse set at 26°C with a 12 hour day/night light regime.

From 11/07/2011 to 09/08/2011, every week one replicate, comprised of all five crosses, was moved to the growth chamber for exposure to a constant environment. The reason for moving the plants to the growth chamber was that the levels of light, humidity and temperature were under constant control in the chamber; whereas in the glasshouse, because of forecast variations, these elements may not be constant from day to day. The temperature in the growth chamber was 22°C and the light regime was set at 12 hours light/dark. For limiting the evaporation rates and, therefore, reducing the possible effects of water deficiency on plants, the temperature of the chamber was set on 22°C which was 4°C less than that of the glasshouse.

After one week, A , E , g_s and internal concentration of CO_2 (C_i) of three leaves from each plant were measured using a Li-COR Biosciences portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, Nebraska, USA). It is important to note that the leaf chamber was equipped with an extra light source for measuring light saturated photosynthesis. The photosynthetic photon flux density (PPFD) was measured to achieve light-saturation in almonds. In this regard, the light saturation point was set at $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The external CO_2 concentration was set at $400 \mu\text{mol mol}^{-1}$, temperature was 22°C and air flow rate was 350 mmol s^{-1} . The relative humidity was kept nearly constant throughout the experiment (50-55%).

For each plant, three upper undamaged, fully expanded and healthy leaves were selected for measurement. Practically, all the measurements were performed on the same branch and every measured leaf was tagged with a unique number. After that the tagged branch was separated from the plant for measuring the leaf specific hydraulic conductance of a shoot using the Hydraulic Conductance Flow Meter (HCFM, Dynamax, Houston, Texas, USA). The branch was cut in water to avoid the entrance of air bubbles into the veins and vessels (Figure 3.2). The transient method was used to measure flow rate as a function of pressure as outlined in (Vandeleur et al., 2007) to give the conductance (k). The procedure of measuring with HCFM is described in section 3.2.1.1.

Leaf area A of all the separated leaves was measured with an AM300 Portable Leaf Area Meter and the leaves were dried in the oven. The measured conductance was normalised by dividing by total leaf area to give (L_{shoot}) ($\text{kg}\cdot\text{s}^{-1}\cdot\text{MPa}^{-1}\cdot\text{cm}^{-2}$) (Sack and Holbrook, 2006). Meanwhile, WUE_i were calculated as A/E (Condon et al., 2002).

3.2.1.1 HCFM methodology

The k was measured with a Hydraulic Conductance Flow Meter (Dynamax, Houston, Texas, USA). First, the HCFM was tightly attached to an almond branch (Figure 3.2). A suitably chosen rubber for fitting the branch in the compression fitting (CF), reduced the probability of leakage. However, any leakage in the tubes notably reduces the pressure that can be detected on the monitor. The nitrogen gas flow was regulated by a needle valve (NV) connected to a captive air tank (CAT). This tank, contained degassed water and air, and was either pressurised or depressurised by regulating the NV. The pressurisation rate increased linearly. There were two series of the 8-way manifolds comprised of inlet and outlet manifolds. These two manifolds were connected to pressure transducer 1 (PT1) and pressure transducer 2 (PT2). They were also connected to each other by 6 capillary tubes which varied in diameter. The flow rate was calibrated by passing water through the different capillary tubes between

the two transducers. A dual channel A/D circuit regulates the pressure transducers and records the measurements in HCFM (Vandeleur et al., 2007).

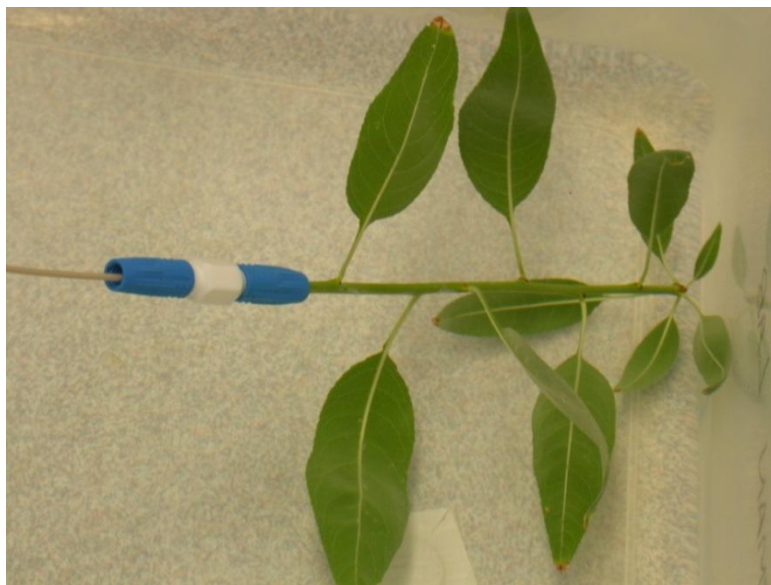


Figure 3.2 After cutting under water condition, the branch is tightly attached to the tube.

3.2.2 Experiment 2

In another experiment, three varieties of a new set of almond trees (Nonpareil, Carmel and Masbovera) were selected in 4 replicates for measuring g_s at field capacity. This experiment was started on 17 May 2013 and was terminated on 21 May 2013. Plants were grown in the same soil conditions as the previous experiment containing coco peat mix (2/3 peat, 1/3 sand) plus slow release fertiliser. The temperature of the glasshouse was set at 26°C with a 12 hour day/night light cycle. Measurements were recorded between approximately 1000 and 1200 h solar time. Although trees had their last fresh leaves of the season, they were not dormant during the experiment. Every second day pots were adequately watered and during 5 days the g_s of leaves was recorded daily using a leaf porometer (Decagon, model SC). The obtained data were statistically analysed in Statistical Analysis Software (SAS Institute Inc. 2004, SAS/STAT, 9.1).

3.2.2.1 Visualizing the post-venous area by Scanning Electron Microscope (SEM)

The internal structures of fully expanded upper leaves collected from the same varieties of almond trees (Nonpareil, Carmel and Masbovera) were imaged by cryo-scanning electron microscopy (cryo-SEM) method at Adelaide Microscopy. Cryo-SEM is an imaging technique for those samples which contain moisture in their tissues. In fact, in this method tissues can be imaged without removing their water. Normally, hydrate samples need to be fixed and dehydrated for visualizing by SEM. Such kinds of microscopes create a high vacuum condition that removes water from hydrated samples. This water removal may distort the morphology of specimens, especially in biological samples (Hwang and Morris, 1991). Freezing method helps to visualize the original structure of biological samples with fewer artefacts compared to conventional sample preparation (Choi et al., 2012, Lee et al., 2009).

Small pieces of leaves (about 1 mm in length) were cut, by using a razor blade, and placed in aluminium planchettes (with a diameter of 3 mm while the diameter and depth of the central cavity were 2 mm and 200 μm , respectively) (Müller and Moor, 1984). Prior to the loading of samples into the cryo-SEM, they were physically fixed by a rapid freezing process in liquid nitrogen. After removing from liquid nitrogen by a scalpel, frozen leaf tissues were loaded, through the airlock system of cryo-chamber, on the specimen stage, and then were clamped between a sample holder (Walther, 2003, Bastacky et al., 1995). With a cold knife which was integrated to the chamber, samples were cleaved for scanning their internal anatomy. After preliminary freezing, there was etching process during which a controlled heating was applied on samples to sublime only ice in tissues. To this aim, the temperature of sample holder was temporarily increased to approximately -90°C . Although, etching process helps to prepare a three dimensional surface texture for imaging, sublimation of too much water may shrink the samples (Walther et al., 1992, Walther, 2003). Due to the high cost of cryo-SEM imaging for each sample, only one sample per treatment was visualized. However, rotating the sample holder in the cryo-chamber allowed the imaging of the samples from different angles; hence, for each section at least 3 veins were clearly imaged and examined.

Most of the resistance for the hydraulic conductivity of a leaf occurs in the D_m area (Mott, 2007) that includes the distance between vascular bundles and stomatal pores (Pickard, 1981). Since measuring the accurate distance of water movement through the mesophyll is still controversial (Ye et al., 2008, Westgate and Steudle, 1985), an index for this path length was calculated in this study. To this aim, D_m was calculated by measuring the horizontal length (x) between vascular bundle and nearest stomata, and the vertical distance (y) from vascular tissue to leaf surface (equation 3.4) (Ocheltree et al., 2012):

$$D_m = \sqrt{x^2 + y^2} \quad \text{equation 3.4}$$

3.3 Results

3.3.1 Experiment 1

There were significant differences in transpiration E , carbon assimilation A , stomatal conductance g_s , internal concentration of CO_2 (C_i) and k normalized to leaf area L_{shoot} (Figures 3.3A, B, C, D and F). For all the measured parameters the observed differences were most significant between Johnston x Lauranne (JxL) and Nonpareil x Lauranne (NxL). Both A and E values in NxL and JxL were significantly different from Nonpareil x Tarraco (NxT), Nonpareil x Vayro (NxV) and Carmel x Tarraco (CxT). For C_i data, only NxL and JxL were significantly different (Figure 3.3F), whereas for A , E and g_s values, NxL was significantly higher than the other 4 crosses. Moreover, L_{shoot} values of JxL were significantly lower than CxT, NxT and NxL. Regarding WUE_i , CxT trees showed significantly higher WUE_i compared to NxV, NxV and NxL (Figure 3.3E). Although, A , E , g_s , L_{shoot} and C_i were not significantly different between CxT, NxV and NxT, the WUE_i of CxT were significantly higher than NxV and NxT.

A highly significant ($P < 0.01$) correlation was observed between L_{shoot} and g_s and also between L_{shoot} and A (Figures 3.4A, B). Although, A was highly ($P < 0.01$) correlated with g_s and C_i , there was a higher correlation between A and g_s compared to A and C_i (Figures 3.4C and D).

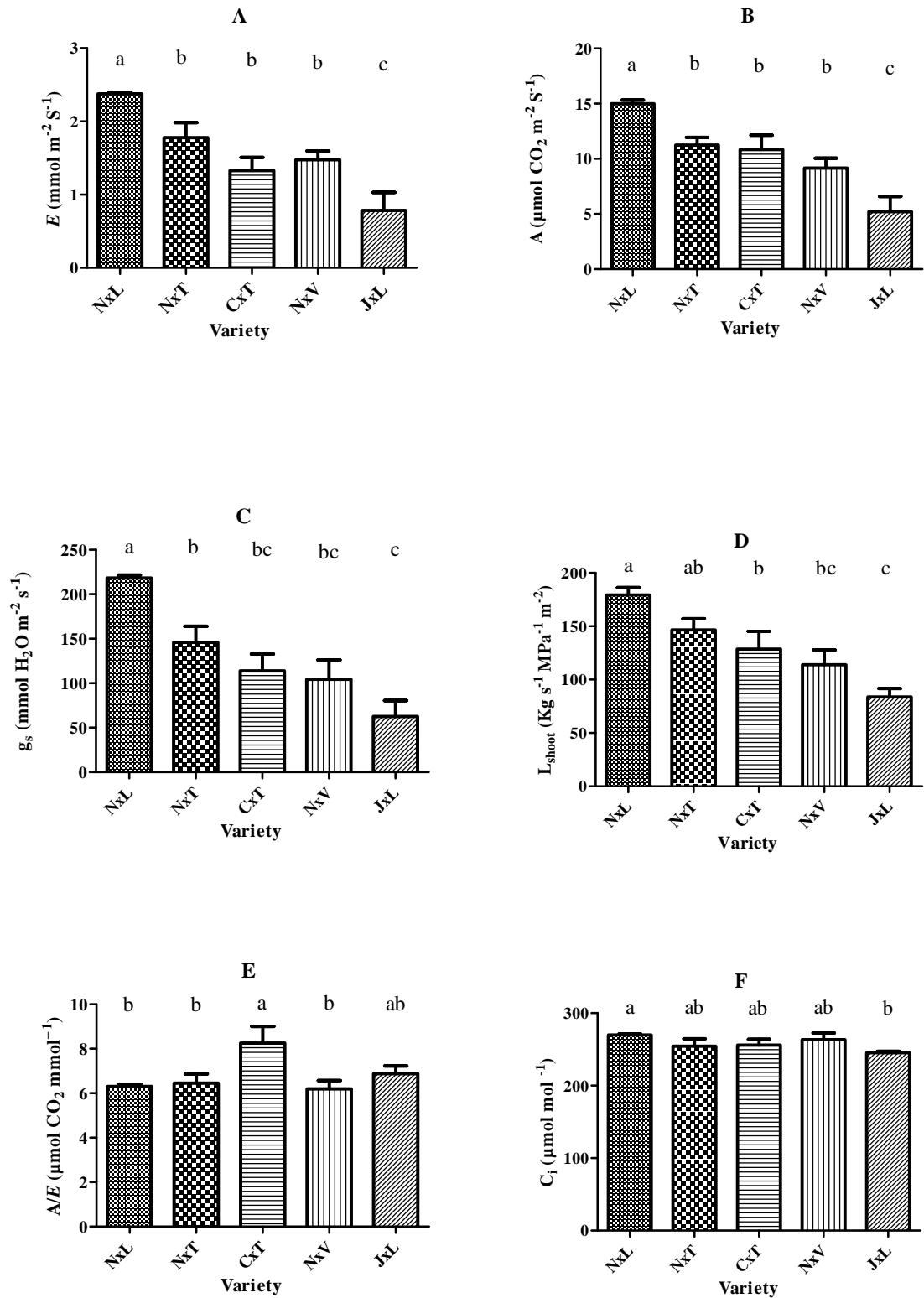


Figure 3.3 Transpiration rate E (A), assimilation rate A (B), stomatal conductance g_s (C), leaf hydraulic conductivity normalized to leaf area L_{shoot} (D), instantaneous water use efficiency WUE_i A/E (E) and internal concentration of CO_2 (C_i) (F) for 5 mixed crosses of almond. Each column represents the average of 4 replicates \pm SE. Different letters indicate statistical differences (Duncan's test; $P < 0.05$).

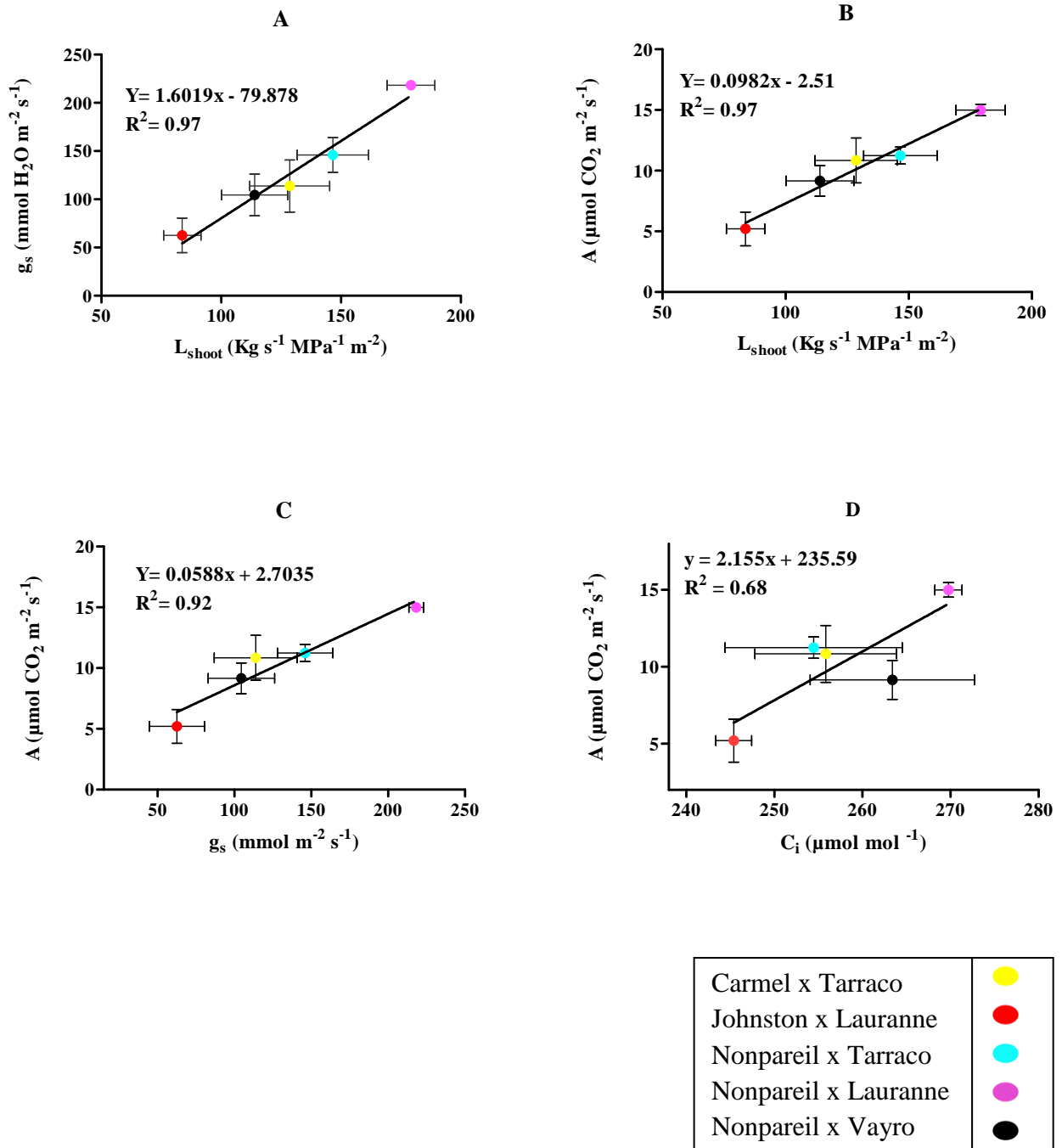


Figure 3.4 The relationships between hydraulic conductance normalised to leaf area L_{shoot} with stomatal conductance g_s (A) and carbon assimilation A (B). The relationships between carbon dioxide assimilation rate A with stomatal conductance g_s (C) and internal concentration of CO_2 (C_i) (D). Error bars represent the average of 4 replicates \pm SE for each point.

3.3.2 Experiment 2

Masbovera showed significantly ($P < 0.05$) lower values of g_s compared to Carmel and Nonpareil (Figures 3.5A). In this respect, g_s values for Nonpareil, which were the nearest to Masbovera, were approximately 2 times greater than Masbovera. However, there were no significant differences in g_s between Carmel and Nonpareil.

Measuring the D_m of 3 veins for each sample in the available images indicated that D_m values for Masbovera were greater than Carmel and Nonpareil (Figures 3.5B, 3.6A and B). Moreover, the appearance of several photos from different angles showed a compact arrangement of mesophyll cells in Masbovera leaves (Figure 3.6C).

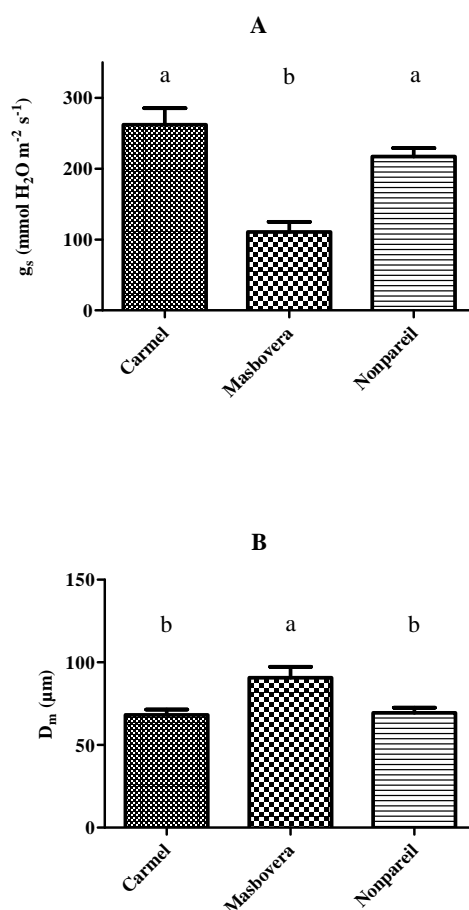


Figure 3.5 The variation of stomatal conductance (g_s) (A) and post venous distance (D_m) (B) for Carmel, Masbovera and Nonpareil. The means \pm SE ($n = 3$ and 8) are shown for D_m and g_s , respectively. Error Bars with the same letter are not significantly different (Duncan's test; $P < 0.05$).

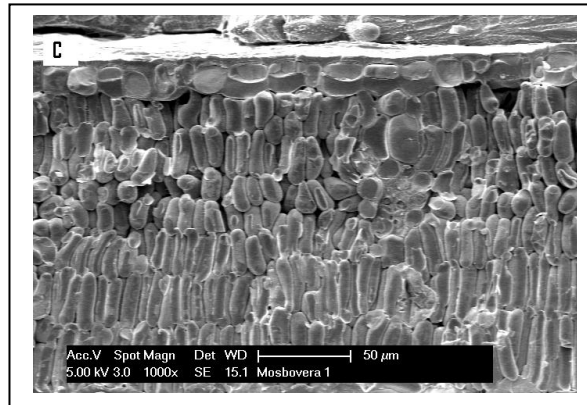
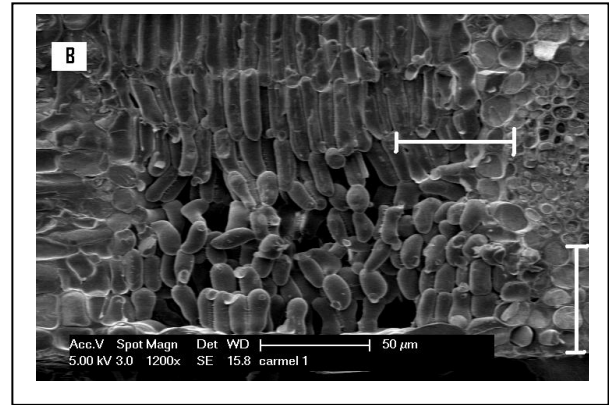
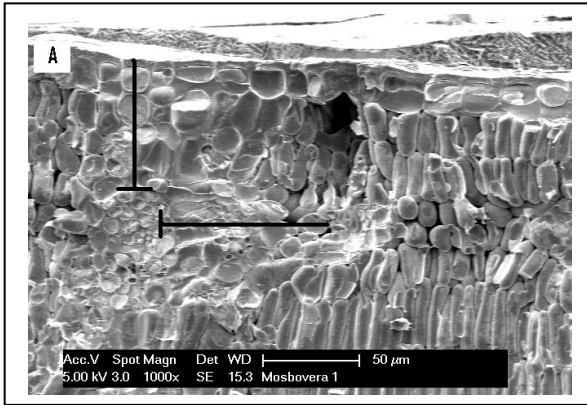


Figure 3.6 Scanning electron microscope images of almond (*P. dulcis*) leaves. Horizontal (x) and vertical (y) distances of vascular bundles from stomata in Masbovera (A) and Carmel (B) varieties. The compact arrangement of mesophyll tissue in Masbovera (C).

3.4 Discussion

The significant decrease in A values from NxL to JxL crosses in the first experiment (Figure 3.3B) was coupled with notable reductions in g_s and C_i (Figures 3.3C and F). According to previous studies, C_i variations indicate that A is probably affected by stomatal limitations (Pena-Rojas et al., 2004, Flexas and Medrano, 2002). Therefore, the lower values of C_i in JxL compared to NxL (Figure 3.3F) imply that A in JxL might be limited by stomatal closure. In other words, stomatal closure is presumably the limiting factor for A (Figures 3.3B and 3.4D). However, the close correlation between A and g_s (Figure 3.4C) can also indicate that stomatal closure might be affected by the photosynthetic capacity of the mesophyll cells (Wong et al., 1979). Based on the theory of stomatal optimality, stomata tend to maintain the C_i at a constant level (Wong et al., 1979, Cowan and Farquhar, 1977, Manzoni et al., 2011). Bearing in mind that the high levels of A lead to a reduction in the partial pressure of C_i , therefore it can be concluded that the higher g_s for NxL (Figure 3.3C) might be the result of its higher A in comparison with other almond crosses (Figure 3.3B and 3.4C) (Wilson et al., 2000). In such conditions, stomata need to open to let in more CO_2 to compensate for the reduction in C_i (Yu and Wang, 1998).

According to previous reports (Sack and Holbrook, 2006, Brodribb et al., 2007), the higher A in NxL is presumably due to its higher L_{shoot} in comparison with other crosses (Figure 3.3D and 3.4B). In fact, the higher values of L_{shoot} in NxL trees indicate that the capacity of the leaf vascular system to supply water for photosynthetic mesophyll cells is probably higher than CxT, NxV and JxL (Figure 3.3D and 3.4B) (Sack and Holbrook, 2006). Thus, it might be that L_{shoot} indirectly affected g_s by limiting A (Figures 3.4A). In this regard, several studies demonstrate the close correlation between L_{shoot} and g_s (Cochard, 2002, Brodribb and Feild, 2000, Meinzer, 2002, Mencuccini, 2003, Addington et al., 2004). Such variations in L_{shoot} are probably due to the anatomical differences between various genotypes (Schreiber et al., 2011, Sack and Frole, 2006).

In the first experiment, the highest and the lowest values of L_{shoot} , A , E , g_s and C_i were observed between NxL and JxL, which both are the progenies of Lauranne (Figures 3.3A, B, C, D and F); hence, comparing water relation parameters between Nonpareil and Johnston might demonstrate even more differences. Moreover, Figure 3.3E shows that the lowest values of WUE_i were belong to Nonpareil progenies (NxL, NxT and NxV). Although, NxL and JxL represented the most significant differences for both A and E (Figures 3.3A and B), their WUE_i were not significantly different (Figure 3.3E). On the other hand, the highest values of WUE_i were observed in CxT, in which A and E were not significantly higher and lower compared to NxL and JxL, respectively. Such results demonstrate that WUE_i in a plant with high A and E may shows the same values as another plant with relatively lower A and E (Condon et al., 2002).

In the second experiment, Masbovera leaves, in which D_m values were higher compared to Carmel and Nonpareil (Figure 3.5 B), represented significantly lower values of g_s rather than the two other varieties (Figure 3.5A). It is probably because of the higher D_m that increases the extra-vascular resistance in Masbovera leaves. Thus, the higher hydraulic resistance in the mesophyll tissues of Masbovera leaves might lead to a lower A that presumably is the reason for the lower g_s in this variety (Sack and Holbrook, 2006, Brodribb et al., 2007). In contrast with Masbovera, both D_m and g_s values were not significantly different between Nonpareil and Carmel varieties. Several studies in this regard indicated that the spatial arrangement of veins in leaves, which determines the D_m , is highly correlated with k_{leaf} , g_s and A (Sack and Frole, 2006, Ocheltree et al., 2012, Brodribb et al., 2007).

Moreover, SEM images revealed that palisade mesophyll layers in Masbovera leaves were more compacted in comparison with the other varieties (Figure 3.6B and C). Such compact arrangement of mesophyll cells might also be the reason for the lower g_s in Masbovera compared to Carmel and Nonpareil. It is previously reported that a compact mesophyll tissue leads to a lower g_m that may reduce A (Pavlovic et al., 2007, Hwang and Morris, 1991, Dacey, 1980, Tomás et al., 2013). Subsequently, g_s may be limited in response to the reduction of A

(Flexas et al., 2007). Related studies on peach (Syvertsen et al., 1995), tobacco (Evans and Loreto, 2000) and bean (Singsaas et al., 2003) demonstrated high correlations between g_m , g_s and A (Flexas et al., 2007, Flexas et al., 2012). Thereby, compact mesophyll tissue limits the amounts of water loss during the hot and dry summers of Mediterranean climates. Related studies on olive trees showed that compact palisade mesophyll layers protect the leaves against extra water loss (Bacelar et al., 2004).

However, there are some reports that the thickness of palisade mesophyll in leaves can also be increased by age (Xie and Luo, 2003, Kositsup et al., 2010). For minimizing errors between young and old leaves, fully expanded upper leaves were collected for this experiment. Nevertheless, data obtained in this section (cryo-SEM imaging) were deducted only from three veins of one sample for each variety.

For future experiments, it remains to be seen if genotypes with shorter post venous pathways are more or less water use efficient. Moreover, measuring $\Delta^{13}C$ and connecting that with WUE_i and leaf anatomical parameters can help to achieve a more accurate insight about water relations in almond leaves.

CHAPTER FOUR

General Discussion

The ever increasing demands on limited water supplies and the high cost of water in different parts of the world, highlights the importance of water use efficiency, particularly for irrigation purposes (Clemmens and Molden, 2007). According to previous reports, two basic solutions are suggested for improving WUE; first is improving water management by adopting effective irrigation methods. The second is breeding new varieties and genotypes which are more water use efficient (Condon et al., 2004). In this study, both solutions for improving WUE in almond orchards were investigated.

To achieve effective irrigation scheduling (first solution), the WUE of almond orchards under different irrigation strategies was examined in Chapter 2. Regardless of irrigation strategy, kernel yield was reduced with irrigation equal to 70% ET_c or less. These results were in agreement with previous studies that applied water deficits below a threshold of -2.0 MPa severely reduce the kernel yield in almond orchards (Romero et al., 2004c, Shackel, 2007). In fact, applying 85% ET_c of full irrigation (100% ET_c) did not adversely impact kernel yield, and resulted in a 15% water saving. However, because of totally different weather conditions between 2009-2010 and 2010-2011 seasons, the effects of mild water deficit on almond orchards over two consecutive seasons remains unclear. Thus, the results obtained from the second season demonstrated that water status and the physiology of trees can be strongly affected by climatic conditions (Jaoude et al., 2013).

In 2009-2010, ΔTC was more sensitive to the quantity of water applied rather than to the type of deficit irrigation strategies (SDI or RDI). Kernel yield on the other hand was slightly lower in RDI 70% ET_c compared to SDI 70% ET_c ; probably because the kernel filling period occurs in late summer, whereas the active vegetative phase is in spring (Romero 2004a). This might be the reason for the moderate correlation between kernel yield and ΔTC in 2009-2010. In

other words, the sensitivity of vegetative growth and kernel yield production to water deficit and environmental conditions is different at different times of the season. Nevertheless, the results obtained in 2009-2010 showed that ΔTC can be used as a reliable water status indicator in almond trees. In this regard, ΔTC and MSWP showed higher sensitivity to different water treatments in comparison with g_s and $\Delta^{13}C$. The lower sensitivity of g_s and $\Delta^{13}C$ to water deficit compared to MSWP might be due to the anisohydric behaviour of almond trees (Wartinger et al., 1990, Egea et al., 2011).

In 2009-2010 season, although there was a strong correlation between $\Delta^{13}C$ and kernel yield, $\Delta^{13}C$ was not highly correlated with WUE. Therefore, consistent with previous reports (Seibt et al., 2008), using $\Delta^{13}C$ as a reliable indicator of changes in WUE is not recommended in this study. Seibt et al (2008) reported that measuring $\Delta^{13}C$ to determine the variations in WUE needs independent measurements of gas exchange or environmental conditions (Seibt et al., 2008). In addition to the net losses of carbon and water through the respiration and non-photosynthesis processes, g_m variations may also reduce the correlation between $\Delta^{13}C$ and WUE (McCarthy et al., 2011). Previous studies indicated that different environmental conditions, e.g. water deficit treatments, can induce mesophyll anatomical alterations that may affect g_m in leaves (Seibt et al., 2008, McCarthy et al., 2011, Chartzoulakis et al., 1999). Leaf anatomy may also change between different genotypes (Barbour et al., 2010, Evans, 1999). Bearing in mind that g_m can strongly affect g_s (Evans, 1999, Flexas et al., 2012), the lower g_s in Masbovera compared to Nonpareil and Carmel could be linked to the compact arrangement of mesophyll cells and lower D_m in Masbovera compared with the two other varieties. However, the lower g_s may not necessarily lead to a higher WUE_i in Masbovera compared to Nonpareil and Carmel (Condon et al., 2002). In this regard, CxT trees did not represent the lowest g_s values but their WUE_i was higher than the other genotypes.

For future work, measuring the WUE_i of Masbovera trees, or comparing it with $\Delta^{13}C$ values would be worthwhile. However, similar to other plant species, the differences in WUE_i between different almond varieties are not fully understood (Tambussi et al., 2007, Xu and Li,

2006, Condon et al., 2004). Previous work has indicated the advantages of using more water efficient rootstocks for improving water use efficiency (Romero et al., 2006). Based on these results, Masbovera and CxT could be examined in future studies for use as rootstocks in water deficit conditions.

Those experiments outlined in Chapter 3 were related to the second mentioned solution for improving WUE; identifying new genotypes which are more water use efficient. Regarding the first solution (Chapter 2), Since WUE and water status indicators were more affected by the level of water applied rather than the irrigation strategy, applying a variety of irrigation levels on trees would be worthwhile to investigate. However, similar studies recommended that combining both methods for improving WUE (improving water management and selecting water use efficient genotypes) is more effective compared to applying them individually (Parry and Lea, 2009, Condon et al., 2004). In this respect, applying a wide range of irrigation levels on CxT and Masbovera trees could be examined for future work. Meanwhile, in regions where severe water stress occurs irrigating CxT and Masbovera with 55% ET_c or less would probably result in an improvement in water productivity with yield penalty.

The results obtained in this study was in agreement with other reports that plant WUE (WUE_i and gross production WUE) can change depending on both genotype and environmental conditions, e.g. deficit irrigation (Flexas et al., 2012, Barbour et al., 2010, Condon et al., 2002).

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