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STUDIES ON SOME ECOPHYSIOLOGICAL, METABOLIC AND AGRONOMIC ASPECTS OF TREE NUTS

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General introduction

Plants harvest the energy of sunlight by converting light energy to chemical energy. The non-structural carbohydrates - NSCs (soluble sugars, mainly sucrose, plus starch), synthesized by the Calvin cycle, are then converted into storage forms of energy and carbon. NSCs play an important role in perennial plants, in particular deciduous trees, by supplying the required energy for frost resistance, bud break and growth of new plant organs at the beginning of the growing season (Myers and Kitajima, 2007; Naschitz *et al*., 2010), and moreover to protect themselves from environmental abiotic and biotic stress (Zwieniecki and Lampinen, 2015). Thus the vegetative life of any plant can be described as nonstop balance in acquiring, transferring and storing energy and these dynamics have been reported as indicators of carbon source – sink relationships (Gough *et al*., 2009).

In both natural and agricultural conditions plants are often exposed to environmental stresses such as drought, high temperature, cold, heavy metals and high salinity that can impair plants growth and their productivity (Anjum *et al*., 2011). In many agricultural areas drought probably is the most common environmental stress; furthermore the fast climate changes suggest an increase in aridity particularly in Mediterranean and temperate zones and, as consequence, a large reduction of agricultural water availability (Chaves *et al*., 2009; Kiparski and Gleik, 2003). Recently strategic change in irrigation management is taking place to save water (deficit Irrigation Management) and currently it is accepted the necessity to maximize yield per unit area minimizing the consumption of water (Fereres *et al*., 2007).

Drought stress involves several morphological, physiological and biochemical aspects of the plants (Robichaux, 1984; Chaves *et al*., 2009; Anjum *et al*., 2011). The first response of plants to water deficit is to limit leaf transpiration by stomatal closure that causes a decline of leaf intercellular CO_2 concentration and

thus the photosynthetic activities (Jones, 1985; Flexas and Medrano, 2002b; Grassi and Magnani, 2005); it has also been reported that in water scarcity condition, there is a down-regulation of biochemical capacity to assimilate CO_2 that can be observed as a reduction of the maximum velocity of Rubisco for carboxylation (Flexas *et al*., 2004; Diaz-Espejo *et al*., 2007).

In the last years an increasing interest in the drought resistant crop such as pistachio, which can produce sustainably in arid and semiarid area, is taking place. In Italy pistachio cultivation is mainly located in Sicily (Barone and Marra, 2004) and it represents just 1% of the world production (FAO, 2013); nevertheless it is an important economic product for the area and appreciated worldwide for its quality characteristics, as the intense green colour, nutrients content and organoleptic properties (Di Marco, 1987; Giuffrida *et al*., 2006; Gentile *et al*., 2007). In California, one of the most important producers (FAO, 2013), orchards are irrigated; on the contrary in Sicily pistachio cultivation is mostly rainfed, even though irrigation systems are installed in some new orchards.

Pistachio is considered a drought and saline tolerant species (crane and Iwakiri, 1981; Behboudian *et al*., 1986; Goldhamer *et al*., 1987); but several studies have reported a positive influence of irrigation on photosynthetic assimilation rate (de Palma and Novello, 1998; Gijón *et al*., 2011), yield and constant production (Goldhamer and Beede, 2004; Goldhamer *et al*., 2004, 2005). Nevertheless, as consequence of a large reduction of agricultural water use, in the recent years studies focused attention on the response of this species to deficit irrigation in specific phenological stages of nut development aims to not reduce the yield; the water volumes reported in literature (varying from 800 to more than 1000 mm) are no more sustainable in most areas.

Aim of this study was to improve knowledge about the physiological and agronomic behavior of mature pistachio trees (*Pistacia vera* L., cultivar Bianca) in a typical Mediterranean environment characterized by low water availability

for agricultural purposes. Moreover we focused attention on carbohydrates metabolism in mature walnut trees (*Juglans regia* L., cultivar Chandler) in California area that represents one of the world's most important producers. In the last years several researches have been reached out aim to understand how walnut trees respond to variable environmental condition, such as the increment of the temperature and the water scarcity. Surely understanding of carbohydrate management inside trees may be of key importance to crop production predictions, determination of plant stress level and phenology.

The aims of the thesis were studied into four different experiments.

In details, the first experiment deals with physiological behavior of pistachio tree under three different levels of water supply, in order to improve the irrigation management in an environment characterized by low water availability. Furthermore we investigated if irrigation can improve yield and dampen the alternate bearing that characterize this species.

In the second experiment challenge was to obtain knowledge concern the photosynthetic response of pistachio leaves to CO_2 concentration under different irrigation treatments; specifically aim was to obtain a quantitative seasonal estimate of photosynthetic parameters during the growing season and to study the influence of the water status on the photosynthetic capacity of this species.

The third study deals with some characteristics of Sicilian fresh pistachio nuts, in terms of chlorophylls content (a + b) and volatile composition. Particularly we focused attention on the influence that the levels of water stress reached by trees at the harvest time had on these quality parameters.

Finally in the fourth experiment, attention was focused on the seasonality of carbohydrates concentration (soluble sugars plus starch) in several tissue organs in mature walnut trees (*Juglans regia* L.). Aim was to investigate the dynamics of non-structural carbohydrates that have been considered indicators of carbon source–sink relationships.

Experiment 1

Effect of different irrigation regimes on ecophysiological parameters of pistachio tree (*Pistacia vera* L.)

1. Introduction

In both natural and agricultural conditions plants are often exposed to environmental stress such as drought, high temperature, cold, heavy metals and high salinity that can impair plant growth and productivity (Anjum *et al.*, 2011). Drought probably is the most common environmental stress that involves several morphological, physiological and biochemical aspects of the plants (Robichaux, 1984; Chaves *et al*., 2009; Anjum *et al.*, 2011). Photosynthetic activity and cell growth are among the primary processes influenced by drought stress (Chaves, 1991). The effects can be direct, as the reduction of CO_2 availability caused by limitations through the mesophyll and stomata (Flexas *et al.*, 2004) or the alterations of photosynthetic metabolism (Lawlor and Cornic, 2002), or can be indirect and caused by oxidative stress processes (Chaves *et al*., 2009).

Irrigated agriculture is practiced in many areas of the world. Climate changes suggest an increase in aridity in Mediterranean and temperate areas (Chaves *et al*., 2009) and there is uncertainty about what will happen in the next future (Fereres and Soriano, 2007); therefore optimization of agricultural water use is required (Kiparski and Gleik, 2003). When water is limiting, farmer's goal should be to maximize yield per unit area minimizing the consumption of water, in accord to basic principles of sustainability. The application of water below the evapotranspiration (ET) requirements is called Deficit Irrigation (DI) and it is an optimizing strategy under which crops are allowed to sustain some degrees of water deficit and yield reduction. The aim of this technique is to increase water

use efficiency, either by reducing water supplied by irrigation or by eliminating the least productive irrigations. To quantify the proper level of deficit it is necessary to define the full crop ET requirements. Several are the techniques of deficit irrigation such as continuous deficit irrigation (CDI), regulated deficit irrigation (RDI), low frequency irrigation (LFI) and partial rootzone drying (PRD). RDI is a model of water management by imposing periods of water deficit in specific phenological stages with no, or low, reduction in yield (Chalmers *et al.*, 1981; Mitchell and Chalmers, 1982; Behboudian and Mills, 1997; Marsal and Girona, 1997; Guerrero *et al.*, 2006).

Pistachio is a drought and saline tolerant nut tree species native to Western Asia and Asia Minor where it's still found growing wild (Crane and Iwakiri, 1981; Behboudian *et al.*, 1986; Goldhamer *et al.*, 1987). In pistachio, has been already reported that irrigation has a positive influence on yield (Polito and Pinney, 1999; Goldhamer, 2005; Ak and Agackesen, 2006; Gijón *et al.*, 2009), improves quality in terms of higher percentage of splitted nuts (Goldhamer and Beede, 2004; Goldhamer, 2005), reduces the alternate bearing (Kanber *et al.*, 1993; Goldhamer, 1995) improving flower buds retention in the "on year" (Marra *et al.*, 1997, 2009) and CO_2 assimilation rate (De Palma and Novello, 1998; Gijón *et al.*, 2011). Further studies have been reported variable physiological behavior of pistachio throughout the growing season. Depending on the phenological stages, a strong influence of water stress on gas exchange, specifically during the period of kernel growth, has been reported (Gijón *et al.*, 2011; Marino, 2012; Galletta, 2014). Goldhamer *et al.* (1987) found that in mature pistachio trees growing in shallow soil, a reduction in irrigation of 50% of the Et_c (crop evapotranspiration) during Stage II (shell hardening period) had no effect on final yield. Later Goldhamer and Beede (2004) reported that a reduction in irrigation of 50% of the Et_c in both Stages I (shell expansion period) and II did not reduce total yield and increased the percentage of shell splitting. Moreover, it has been reported that in pistachio tree RDI in the period of shell hardening and postharvest is a viable irrigation strategy to save water while maintaining high yield (Goldhamer and

Beede, 2004; Guerrero *et al*., 2006; Gijón *et al*. 2009, 2011). Similar results have been reported in successful experiments reached out in other species such as pear (Mitchell *et al.*, 1989), citrus (Domingo *et al.*, 1996; Gonzalez-Altozano and Castel, 1999; Goldhamer and Salinas, 2000), apple (Ebel *et al.*, 1995), apricot (Ruiz-Sanchez *et al.*, 2000), wine grapes (Bravdo et al., 2003; McCarthy *et al.*, 2002), olive (Moriana *et al.*, 2003) and almond (Goldhamer and Viveros, 2000). In Italy pistachio cultivation is mainly located in Sicily (Barone and Marra, 2004) and the production represents just 1% of the world production (FAO, 2013). However it is an important economic product for the region and appreciated worldwide for its quality characteristics (Di Marco, 1987; Giuffrida *et al.*, 2006; Gentile *et al.*, 2007). In Sicily pistachio has been traditionally cultivated in dry and marginal areas characterized by the unsuitable conditions for applying modern management system (Barone *et al*., 1985), but in the last years new irrigated orchards have been developed. Caruso *et al*. (1996) showed that low water volumes (1000-1500 m^3/ha) might improve crop yield and reduce alternate bearing.

Up to date, few studies have been reported the effect of different irrigations regimes on pistachio production in the Mediterranean area (Caruso *et al*., 1996; Gijón *et al*., 2009; Memmi *et al*., 2016). In order to improve the irrigation management, especially in condition of limited water resources, a study has been developed to investigate the physiological and productive response of mature pistachio trees to three different levels of water supply.

2. Materials and methods

2.1 Site of the research and plant material

The trial was carried out during two years (2014 and 2015) in a commercial pistachio orchard located at Caltanissetta, Italy (37°26'02" N, 14°03'12" E; altitude 360 m) on 30-year-old trees *Pistacia vera* L. cultivar Bianca, grafted on *P. terebinthus* L. rootstock (Fig. 2.1), which is the most common used in Sicily (Caruso *et al.*, 1996; Ferguson *et al.*, 2005). The trees were spaced 6.5 x 4.5 m apart (340 trees ha^{-1}).

The orchard was managed following the standard cultural practices as commonly recommended to the fanners were adopted for the study.

The climate at the experiment site is typically Mediterranean, characterized by long, dry and hot summers and mild winters with irregular rainfalls which are mostly distributed outside a four/five-months summer drought period (Cartabellotta *et al.*, 1998). The physical composition of soil in the site was: sand 33.8%, silt 15.1% and clay 51.1%.

Fig. 2.1 – View of the experimental site (by Google Earth)

Water was supplied to the trees by two pressure compensating integral drip-lines per row, with emitters spaced 80 cm along the pipe.

The irrigation treatments were as follows:

- T_0: rainfed conditions (control);
- T_1: 50 mm of water supplied (by 1.6 L h^{-1} emitters);
- T_2: 100 mm of water supplied (by 3.5 L h^{-1} emitters).

In 2014 water was distributed in 7 irrigations throughout the growing season from middle of June to mid-August, while in 2015 water was distributed in 10 times, from the end of June to the middle of August. The total amount of water applied in 2014 was 46.24 mm in T_1 treatment and 101.15 mm in T_2, while in 2015 it was 43.52 mm in treatment T_1 and 95.17 mm in T_2 (Tab. 2.1). Irrigation started in both years when the stem water potential values were around -1.3 MPa (around 55 DAFB in 2014 and 60 DAFB in 2015). Three blocks, constituted by three adjacent rows each, were replicated for each irrigation treatment. Three trees for each block were selected for their uniformity in the central row for a total of 9 trees per treatment.

2014			2015		
Day	**T$_1$ (mm)**	**T$_2$ (mm)**	**Day**	**T$_1$ (mm)**	**T$_2$ (mm)**
June 17	4.76	10.412	June 30	2.72	5.948
June 24	4.76	10.412	July 6	2.72	5.948
June 27	8.16	17.85	July 11	2.72	5.948
July 8	8.16	17.85	July 15	2.72	5.948
July 16	8.16	17.85	July 20	5.44	11.896
July 22	8.16	17.85	July 26	5.44	11.896
August 12	4.08	8.925	July 31	5.44	11.896
			August 5	5.44	11.896
			August 10	5.44	11.896
			August 14	5.44	11.896
Total	**46.24**	**101.15**	**Total**	**43.52**	**95.17**

Tab. 2.1 - Irrigation dates and their corresponding amount of water (mm) in the two irrigation treatments (T$_1$ and T$_2$) during the growing seasons in 2014 and 2015.

2.2 Meteorological data

Daily climatic data were acquired from a public weather station (SIAS - Servizio Informativo Agrometeorologico Siciliano), positioned next to the experimental site (37°25'42" N, 14°03'03" E; altitude 350 m. a.s.l., 0.6 km away from the orchard).

To characterize the site were considered the following data: maximum and minimum air temperature (°C), rain (mm), maximum and minimum relative humidity (%), wind speed (m/s) and global solar radiation (MJ/mq).

Crop evapotranspiration (Et$_c$) was calculated using CROPWAT 8.0 model based on the recommended FAO procedure (Allen *et al.*, 1998). The FAO Penman–Monteith equation was used to calculate reference evapotranspiration (ET$_o$) for 2014 and 2015 years.

The daily reference crop evapotranspiration (ET_o) was then used to calculate the crop evapotranspiration as follows:

$$ET_c = ET_o \times K_c \times K_r$$

where the crop coefficient (K_c) was obtained from the literature (Goldhamer, 1995) and it varies with the phenological stage of the crop, while the reduction coefficient (K_r), that takes into account the fraction of ground covered by the crop, was calculated from direct measurements of shaded soil at midday and resulted 0.60 (Tab. 2.2).

The effective rainfalls, portion of rainfall that can effectively be used by trees, were also calculated using the USDA Natural Resources Conservation Service (NRCS) methodology (Obreza and Pitts, 2002; USDA, 1970).

Time	K_c	K_{cr}
April 1-15	0.07	0.04
April 16-30	0.19	0.11
May 1-15	0.41	0.25
May 16-30	0.64	0.38
May 31 - June 14	0.86	0.52
June 15-29	1.09	0.65
June 30 - July 14	1.19	0.71
July 15-29	1.19	0.71
July 30 - August 13	1.19	0.71
August 14-28	1.19	0.71
August 29 - September 12	1.01	0.61
September 13-30	0.66	0.4

Tab. 2.2 - Crop coefficient (K_r) proposed by Goldhamer for pistachio in California area (1995) and crop coefficient corrected (K_r) taking into account the fraction of ground covered by the crop.

2.4 Stem water potential

Plants water status was monitored measuring midday steam water potential (Ψ_{SWP}) with a Scholander pressure chamber (PMS Instrument Co., Corvallis – Oregon, USA).

Measurements were made on two fully expanded shaded leaves per tree (eighteen leaves total), selected in bearing branches and positioned in the middle part of the canopy. One hour before measurement, leaves were covered with transparent film and aluminum foil in order to stop transpiration and equilibrate leaf with stem water potential (Begg and Turner, 1970). Pistachio is a resinous plant, thus a piece of blotting paper was used to determine the end point distinguishing turpentine exudation from xylem water (Ritchie and Hinckley, 1975; Pearcy and Sims; 1994, Gijón *et al.*, 2009). During the growing seasons 2014 and 2015, Ψ_{SWP} was measured 8 times (see table 2.3).

2014		2015	
Day	**DAFB**	**Day**	**DAFB**
May 20	26	May 13	16
June 9	47	May 20	23
June 27	65	June 16	50
July 3	71	June 26	60
July 11	80	July 4	78
July 24	93	July 30	98
August 7	107	August 7	102
August 18	118	August 27	122

Tab. 2.3 - Dates sampling in 2014 and 2015 and their corresponding days after full bloom (DAFB). During the two-year experiment full bloom was April 24 in 2014, and April 27 in 2015.

2.5 Gas exchanges

At the same dates (see table 2.3), from the same trees in which Ψ_{SWP} was measured and at the same time (midday), physiological parameters were

measured. Particularly, maximum net assimilation rate (A_{max}, µmol m^{-2} s^{-1}), stomatal conductance (g_s, mmol m^{-2} s^{-1}) and intrinsic water use efficiency, calculated as A_{max}/g_s ($_iWUE$, µmol CO_2 mol^{-1} H_2O), were measured on two sunlight and fully expanded leaves for each tree selected in bearing branches and in the middle part of the canopy.

Measurements of gas exchanges were made using a portable infrared gas analyzer (CIRAS - 3, PP Systems. Amesbury, Massachusetts, USA) connected to an automatic leaf cuvette (PLC6 (U) PP Systems). Leaf chamber covered an exposed area of 2.5 cm^2; cuvette conditions during measurements were maintained constant: saturating photosynthetic photon flux density (PPFD) at 1500 µmol m^{-2} s^{-1} provided by a LED light unit, temperature at 27 °C, flow at 200 ml min^{-1} and CO_2 concentration at 380 ppm. Readings were taken after a steady-state condition in gas-exchanges was achieved.

2.6 Nut characteristics and yield

At the beginning of the growing season, in both years, were randomly selected 2 branchlets per each tree (eighteen branchlets total). On the selected branchlets the following parameters were monitored throughout the growing seasons: number of infructescences, number of fruits and number of flower buds.

By the number of the flower buds at the beginning and at the end of the growing season was also calculated the percentage of bud that dropped. Furthermore, on fruit samples of the tagged branchlets, were measured fresh and dry weight of nuts (in shell) and kernels; dry weight was measured using a ventilated oven at 40°C until a stable weight was reached.

Harvest was done the 15th of September in 2014 and the 10th of September in 2015. Yield per tree was measured, in terms of fruits in shell; moreover were calculated number of fruits per tree and crop efficiency (CE, yield/trunk cross sectional area).

Phenological stages of pistachio suggested by Goldhamer (1995) were taken into consideration. In details stage 1 covers the whole period of shell expansion from the beginning of the nut growth and finishes when the maximum size is reached (May – beginning of June, in the condition of the experiment); stage 2 covers the period of shell hardening (June – July), and stage 3 is the period of kernel growth until the harvest (August - September).

2.7 Statistical analysis

Data were analyzed using Systat 13.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606). ANOVA and Tukey's multiple range test was performed for comparing the means between treatments; differences were considered statistically significant at $P< 0.05$.

Relationships among parameters were fitted using Sigmaplot 12.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606).

3. Results and discussion

3.1 Meteorological data

The rainfall dates, the amount of water applied for different irrigation treatments and the average temperatures during the trial in 2014 (a) and 2015 (b) are reported in figure 3.1. Temperatures in both years followed the same trend: the maximal temperatures were recorded in August (39°C) in 2014 and in July (41°C) in 2015.

Effective rainfalls during the growing seasons (April – August) were 57.4 mm in 2014 and 80 mm in 2015 (34.6 mm fall in August). The driest month was August in 2014 (0 mm) and July in 2015 (6.6 mm).

The coldest month was January in 2014 (average 5.2 °C) and again in 2015 (average 3.5 °C), while the warmest was August in 2014 (average 34.6 °C) and again in 2015 (average 35.5 °C). Cumulated precipitations were 421.2 mm in 2014 and 682.6 mm in 2015. In 2015 rainfall was definitely higher than the average value (approximately 550 mm) reported for the area where the orchard is located (Cartabellotta *et al.*, 1998). In both years rain was mainly recorded during fall-winter months. In 2014 the rainiest month was March (113.4 mm), while in 2015 it was February (191.2 mm).

Taking into consideration rainfall and the irrigation dates, the total amount of water supplied was in 2014 103.6 mm in T_1 and 158.5 mm in T_2 treatment, and in 2015 it was 123.5 mm and 175.2 mm for T_1 and T_2 treatment respectively. T_0 trees received through rainfalls 57.4 mm in 2014 and 80 mm in 2015.

Using meteorological data, the calculated ET_c was 397.7 mm in 2014 and 426.9 mm in 2015. According to these data in 2014 we applied 26% of the ET_c in T_1 and 36% in T_2 treatment, while in 2015 it was 40% and 48% in T_1 and T_2 treatment respectively. Taking into consideration only rainfalls, T_0 trees received the 14% of the Et_c in 2014 and the 25% in 2015.
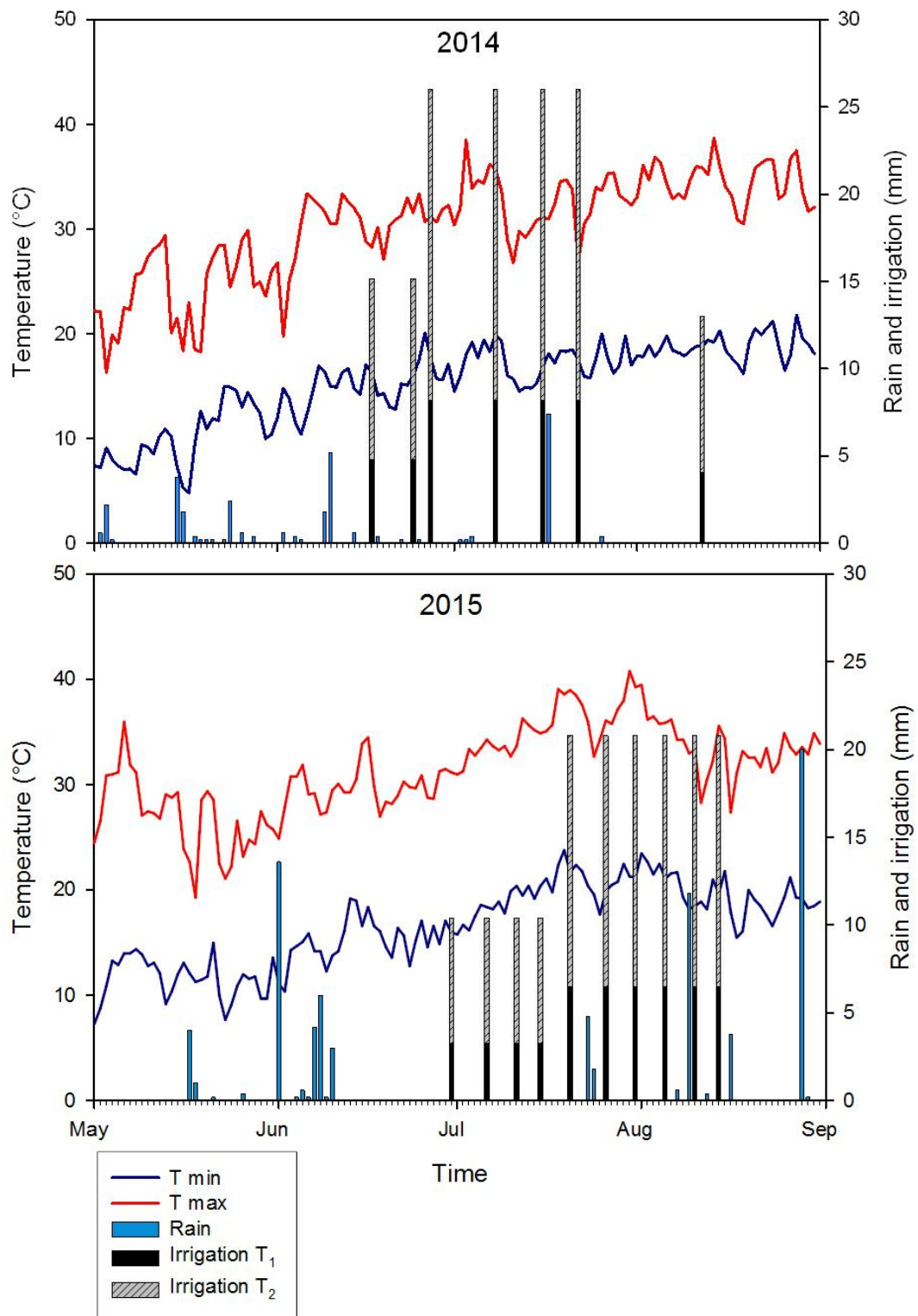
Fig. 3.1 - Rainfall (blue bars), daily air temperature (red and blue lines for maximum and minimum temperatures respectively) and amount of water applied (grey bars) for different irrigation treatments (T_1 and T_2) during the period June – August in 2014 (a) and 2015 (b).

3.2 Stem water potential

Figure 3.2 shows the time course of midday stem water potential (Ψ_{SWP}) in 2014 (a) and 2015 (b) as affected by different irrigation treatments. Patterns were similar and decreasing in midsummer in both years, as also it has been reported for pistachio trees by Goldhamer (2005), Gijón *et al.* (2009) and Memmi *et al.* (2016). In 2014 (Fig. 3.2 a) at the beginning of the experiment (Stage 1 – May) Ψ_{SWP} values were similar (around -0.8 MPa) in all treatments. By 47 DAFB Ψ_{SWP} slightly declined (around -1.2 MPa); similar values in the same stage have been reported in several studies on pistachio (Guerrero *et al.*, 2006; Gijón *et al.*, 2009). Significant differences were found starting from stage 2, 65 DAFB (June) and 71 DAFB (July) when irrigated trees were less stressed compared than the rainfed ones. In July, after rainfall occurred, water stress recovery was observed in T_2 trees and significant differences between treatments were found (-1.4 MPa in T_2, -1.6 MPa in T_1 and -1.8 MPa in T_0). Until 71 DAFB T_2 trees showed Ψ_{SWP} values ranging from -0.8 MPa to -1.5 MPa that represent no water stress values for pistachio as confirmed by any negative effects on tree productivity (Shackel *et al.*, 1994). From mid-July to the end of August, no rainfalls were recorded and irrigation was supplied for only one day; as consequence a very severe decrement of stem water potential was observed in all treatments. The lowest Ψ_{SWP} values were observed at 118 DAFB (the end of August) when all trees were severely stressed (values ranging from -1.9 to -2.3 MPa).

In 2015 pattern was similar to that in previous year (Fig. 3.2 b). In stage 1 and until 60 DAFB (Stage 2 - June) in all treatments were observed Ψ_{SWP} values ranging from -0.8 MPa to -1.3 MPa. A reduction of stem water potential was observed in all trees at 78 DAFB (Stage 2 – mid-July), and Ψ_{SWP} values ranged from -1.9 to -2.2 MPa. In stage 3, at 102 and 122 DAFB (August) significant differences were found between treatments; the lowest Ψ_{SWP} values were recorded at 102 DAFB (-2.2 MPa in T_0, -2 MPa in T_1 and -1.9 MPa in T_2). At 122 DAFB, after several rainfall and irrigation events, Ψ_{SWP} values increased in irrigated trees (-1.7 MPa in T_1 and -1.6 MPa in T_2 respectively); on the contrary

in rainfed trees no any recovery phenomenon was observed, showing Ψ_{SWP} values denoting severe water stress (around -2.1 MPa). The rewatering effect observed in T_1 and T_2 treatments was due to both irrigation and rainfall; in fact rain (16 mm) was not enough to rehydrate T_0 trees.
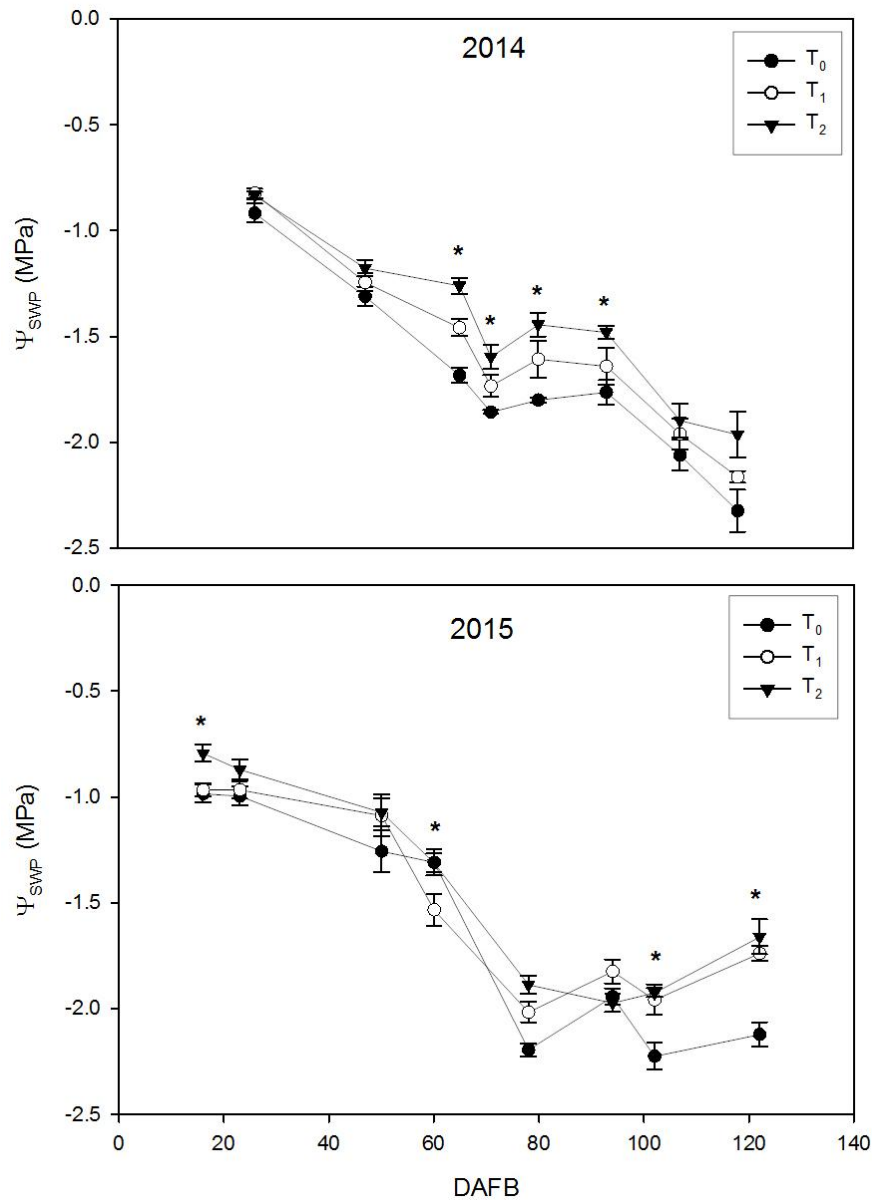
Fig. 3.2 - Time course of midday stem water potential (Ψ_{SWP}, MPa) in pistachio trees during the growing seasons in 2014 (a) and 2015 (b), as affected by irrigation treatments. Closed circles rainfed trees, open circles T_1 treatment and closed triangles T_2 treatment. Values are mean ± S.E. Asterisks denote the dates when significant differences were found among irrigation treatments ($P < 0.05$).

3.3 Ecophysiological measurements

Figure 3.3 shows seasonal trends of maximum assimilation rate (A_{max}) in 2014 and 2015.

In 2014 (Fig. 3.3 a), pattern was decreasing throughout the growing season. In detail at 47 DAFB (beginning of June) was observed a low photosynthetic assimilation (average 12 µmol m^{-2} s^{-1}) related to Ψ_{SWP} values -1.2 MPa. Similar assimilation values have been reported in pistachio trees under non stress conditions and were correlated with the incomplete leaf development and thus to the restricted photosynthetic capacity (Lin *et al.*, 1984; Novello, 1998; Vemmos *et al.*, 1994). At 65 and 71 DAFB (end of June - beginning of July) in T_1 and T_2 treatments were recorded higher A_{max} values with respect to the previous dates; on the contrary rainfed trees at 71 DAFB showed a reduction of 50% of the assimilation rate. At 107 and 118 DAFB (Stage 3 - August), when all trees were severely stressed, the minimum A_{max} values were recorded (average 3 µmol m^{-2} s^{-1} in T_0 trees, 7 µmol m^{-2} s^{-1} in T_1 and 6 µmol m^{-2} s^{-1} in T_2). The deep decrement of A_{max} observed during August was correlated with the severe water stress condition because in that month no rainfall events occurred and water was supplied only in one day. In pistachio has been reported a strong influence of water status on photosynthetic assimilation during the kernel growth phase (Goldhamer, 2005; Gijón *et al.*, 2011); a decreasing pattern in midsummer has been reported in pistachio also in several studies (Gijón *et al.*, 2011; Marino, 2012; Galletta, 2014).

Seasonal pattern of A_{max} in 2015 is shown in fig. 3.3 b. As it was already observed in the previous year, the low values recorded during the first stage, at 23 DAFB (average 8 µmol m^{-2} s^{-1}), were probably related to leaf age rather than to the water stress; trees showed Ψ_{SWP} values around -1 MPa. By 78 DAFB and during the rest of the season, rainfed trees showed a decline of assimilation rates, whereas those irrigated showed a steady and relatively high A_{max} until the end of August. At 122 DAFB (end of August) for T_2 trees were recorded the highest A_{max} values (around 19 µmol m^{-2} s^{-1}), respect to Ψ_{SWP} values around -1.5 MPa

recorded after several rainfall and irrigation events. Overall in 2015 rainfed trees showed throughout the growing season lower A_{max} values with respect to the irrigated ones, which maintained assimilation rate nearly unvaried (around 15 μmol m^{-2} s^{-1}).

These different patterns in two-year experiment, observed also above in stem water potential, were due to the differences in rainfall and irrigation dates. In fact in 2015 water was mostly applied in July and August (Tab. 2.1); moreover August 2014 was the driest month of the year (0 mm) while in August 2015 rainfall occurred.
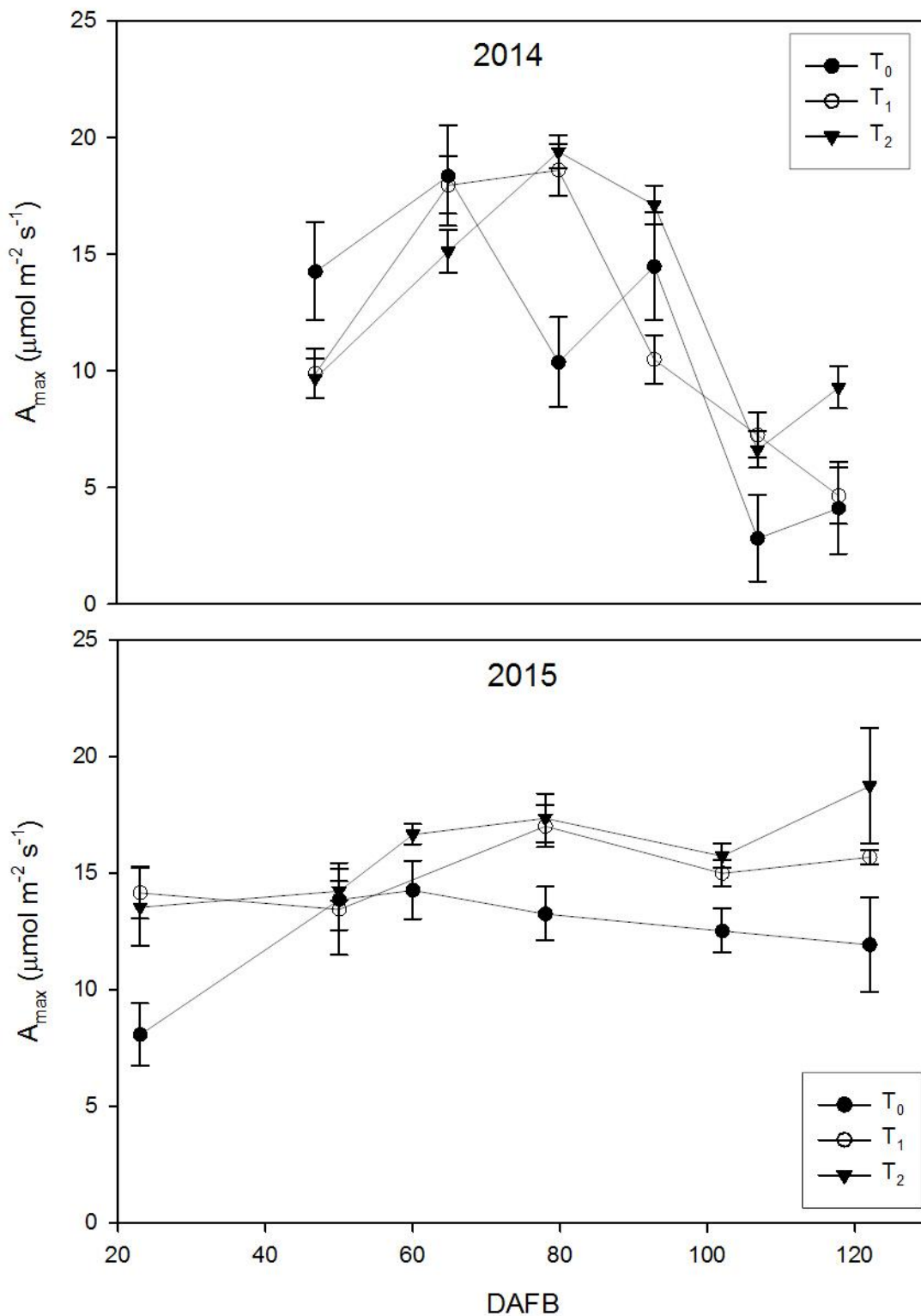
Fig. 3.3 - Time course of maximum assimilation rate (A_{max}, μmol m^{-2} s^{-1}) during the growing season in 2014 (a) and 2015 (b), as affected by irrigation treatments. Closed circles rainfed trees, open circles T_1 treatment and closed triangles T_2 treatment. Values are mean $\pm$ S.E.

In figure 3.4 is shown the positive and significant relationship found between maximum assimilation rate (A_{max}) and stomatal conductance (g_s) for 2014 and 2015 ($R^2 = 0.66{***}$). The exponential curve showed that assimilation increased up to g_s values of around 350 mmol m^{-2} s^{-1}, when the maximum values were reached (almost 20 µmol m^{-2} s^{-1}). From this point the relationship became linear and assimilation did not increase even though were reached g_s values around 800 mmol m^{-2} s^{-1}. Thus 350 mmol m^{-2} s^{-1} may indicate a partial stomatal control as it has been reported in pistachio by Gijón *et al.* (2009) and in other drought tolerant species as olive (Moriana *et al.*, 2002).

The relationship found between intrinsic water use efficiency ($_i$WUE) and g_s for 2014 and 2015 data is shown in figure 3.5 ($R^2 = 0.53{***}$). The highest $_i$WUE values measured (ranging from 0.08 to 0.12 µmol CO_2 mol^{-1} H_2O) were related to g_s values ranged from 100 to 200 mmol m^{-2} s^{-1}; up to g_s values of 300 mmol m^{-2} s^{-1} water use efficiency started decreasing; so this value may indicate a threshold above which pistachio leaves lost water by transpiration without increasing assimilation rate.

To clarify the response of pistachio tree to water status, were examined the relationships between A_{max}, g_s and Ψ_{SWP}, taking into consideration also the stage of nut development reported by Goldhamer (1995). The relationship between A_{max} and Ψ_{SWP} (Fig. 3.6) was poor when only the data from stage 1 was considered (not significant). When both stages 2 and 3 were taken into consideration, was found a significant correlation between these two variables ($R^2 = 0.43{**}$). In stage 2 were observed the highest assimilation rate values throughout the growing seasons (around 20 µmol m^{-2} s^{-1}) related to stem water potential values ranging from -1.1 MPa to -1.6 MPa. In stage 1, the low A_{max} values recorded were related to an incomplete development of the leaves rather than a water stress condition. Finally, in stage 3 it was clear the strong influence that water status had on photosynthetic assimilation; thus in that stage water scarcity results to be the main limitation to the photosynthesis (assimilation rates below 5 µmol m^{-2} s^{-1} and Ψ_{SWP} values ranging from -2 MPa to -2.5 MPa). These

data are in accordance with those reported by Gijón *et al.* (2011) that found the same relationship and reported the highest assimilation rate in stage 2, even though their values were higher compared to those observed in this experiment.

A significant relationship between g_s and Ψ_{SWP} (Fig. 3.7) was found only in stage 3. In stage 2, at the same level of water status was observed high variability of g_s (ranged from 200 to 800 mmol m^{-2} s^{-1}). A weak linear relationship was found in stage 2 by Gijón *et al.* (2011) and they also reported in this stage the highest g_s values (up to 700 mmol m^{-2}s^{-1}). Finally in stage 3 the low g_s values recorded, ranging from 50 to 200 mmol m^{-2} s^{-1}, were related to low Ψ_{SWP} values (-1.5 MPa to -2.5 MPa). A similar decrement of g_s correlated to water stress has already been reported in pistachio trees by De Palma and Novello (1998), Guerrero *et al.* (2006) and Gijón *et al.* (2009).
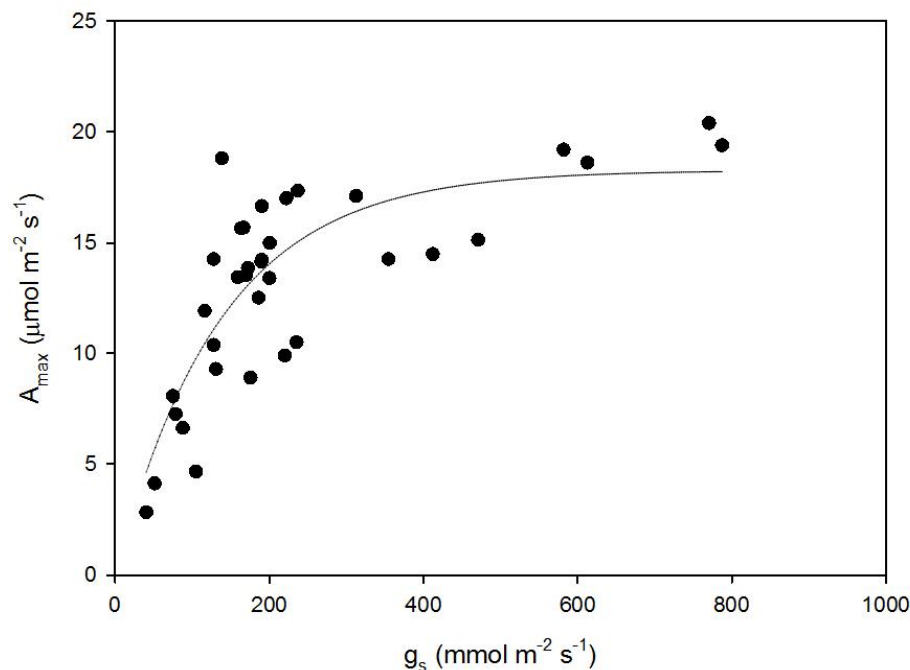
Fig. 3.4 - Relationship between maximum assimilation rate (A_{max}, μmol m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}) using data from 2014 and 2015. The best fit relationship was obtained using an exponential rise to maximum function f= a*(1-exp(-bx)). The parameters are a= 18.27, b= 0.0073; R^2=0.66. P< 0.0001. Values are data points.
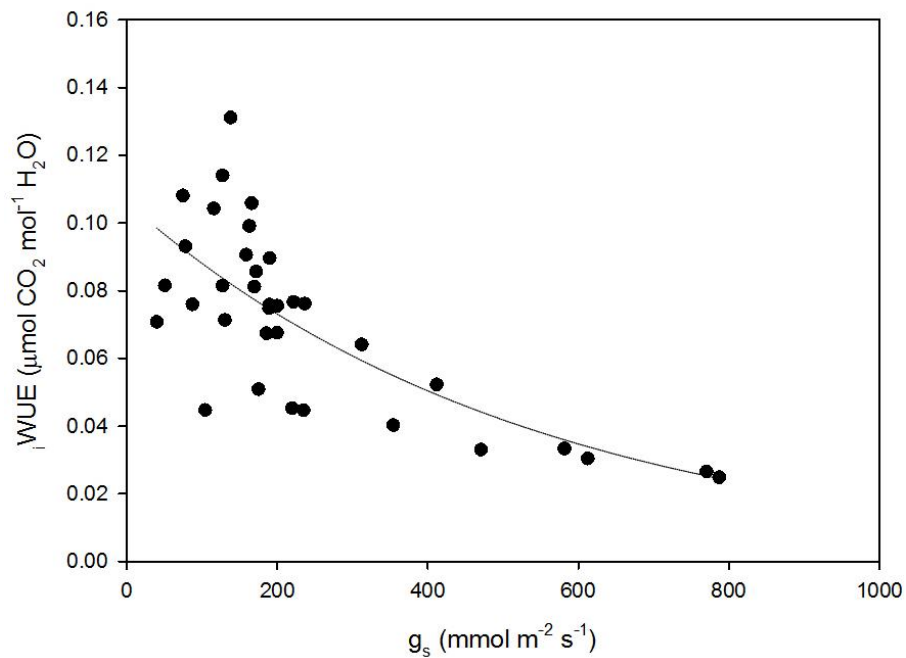
Fig. 3.5 - Relationship between stomatal conductance (g_s, mmol m^{-2} s^{-1}) and intrinsic water use efficiency ($_i$WUE, μmol CO_2 mol^{-1} H_2O) using data from 2014 and 2015. The best fit relationship was obtained using an exponential decay function f= a*exp(-bx). The parameters are a= 0.1062, b= 0.0019; R^2= 0.53. P< 0.0001. Values are data points.
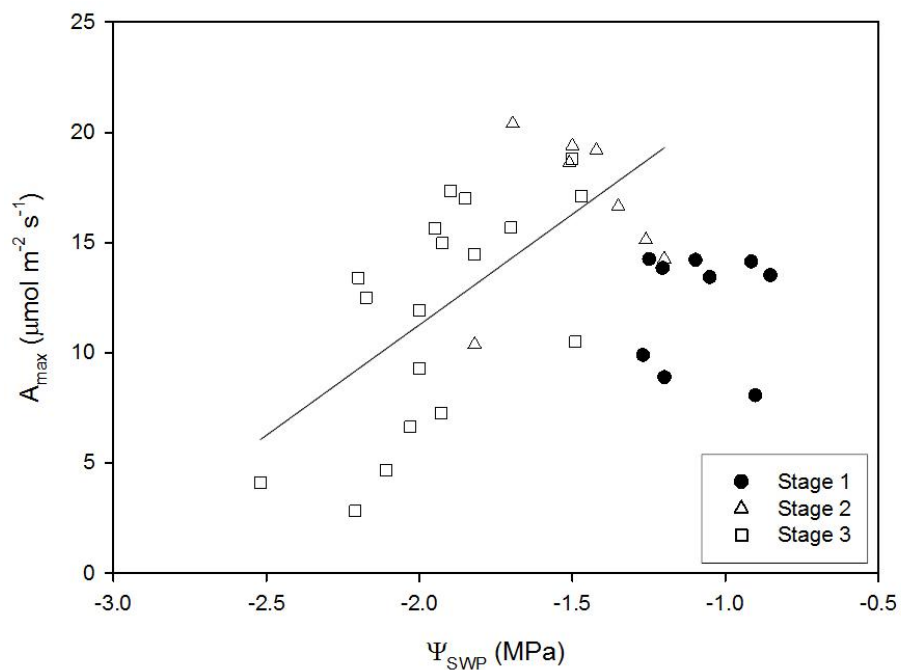
Fig. 3.6 - Relationship between maximum assimilation rate (A_{max}, μmol m^{-2} s^{-1}) and midday stem water potential (Ψ_{SWP}, MPa) as affected by different phenological phases (data 2014 and 2015) The best fit relationship was obtained using a linear function f = y0+ax. The parameters are y0= 31.35, a= 10.03; R^2=0.43. P< 0.01. The relationship in stage 1 is not represented because not significant. Values are data points.
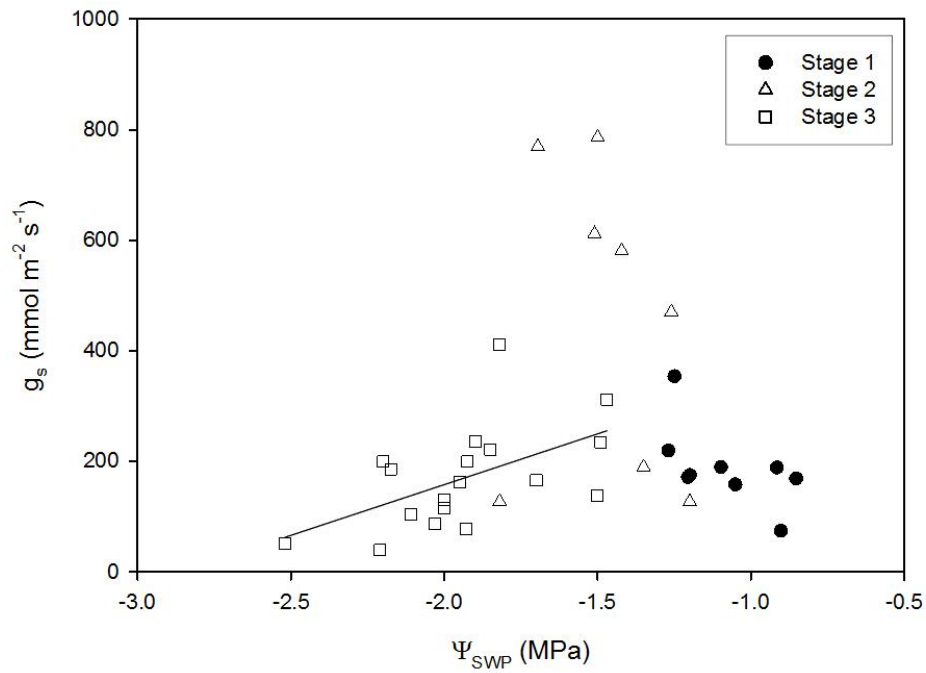
Fig. 3.7 - Relationship between stomatal conductance (g_s, mmol m^{-2} s^{-1}) and midday stem water potential (Ψ_{SWP}, MPa) as affected by different phenological phases (data 2014 and 2015) The best fit relationship was obtained using a linear function f = y0+ax. The parameters are y0= 525.53, a= 183.55, R^2= 0.29. P< 0.01. The relationships in stages 1 and 2 are not represented because not significant. Values are data points

3.4. Nut yield and flower buds drop

Table 3.1 shows the crop parameters as affected by irrigation treatments.

Irrigation had not a significant effect even if, as concerning yield, T_2 trees showed a slightly higher value (around 6 kg in T_0 compared to 7.5 kg in T_2 treatment) and the same occurred as concerning the number of nuts per tree. Significant differences were found between years on yield, crop efficiency and weight of the nuts. Specifically in 2015 yield was higher compared than in 2014 (7 kg and 5 kg respectively), while the weight of the nut was significantly higher in 2014 (1.2 g in 2014 compared to 0.8 g in 2015).

Although the positive influence of irrigation on yield is well known in pistachio trees (Polito and Pinney, 1999; Goldhamer, 2005; Ak and Agackesen, 2006; Gijón *et al.*, 2009), Carbonell-Barranchina *et al.* (2015) reported negligible effects of irrigation on the nuts weight in cultivar Kerman. In pistachio trees, and in general in nut species, irrigation seems to have a positive influence on yield, in terms of number of nuts per tree, rather than kernel dry matter accumulation (Goldhamer *et al.*, 1984; Monastra *et al.*, 1997; Gijón *et al.*, 2009).

Such behavior can also be related to the high variability of yield among trees (Johnson and Weinbaum, 1987), to the effect of tree's maturity (Obeso, 2002), environmental factors (Monselise and Goldschmidt, 1982) and genetic dissimilarity among trees (Wood, 1989; Garner and Lovatt, 2008). Furthermore has been reported that to capture the true behavior of a species, studies should take place over a minimum of six years of observation (Ferguson *et al.*, 2002; Rosenstock *et al.*, 2010).

Flower buds drop was high in both years. Rainfed trees showed a buds drop of 100% and 74% in 2014 and 2015 respectively; in T_1 trees it was 90% in 2014 and 83% in 2015, while in T_2 treatments it was 97% and 72% in 2014 and 2015. Analysis revealed a not significant effect of irrigation treatments on this parameter, probably due to the low amount of water applied in this experiment.

Finally, no differences were found between years as concerning the alternate bearing. Several studies reported the singularity of this phenomenon in pistachio,

that generally shows a large variability among trees (Johnson and Weinbaum, 1987) related to their genetic dissimilarity (Wood, 1989; Garner and Lovatt, 2008). Rosenstock *et al.* (2010) reported that during the "on year" a greater percentage of trees are "on" but there are also trees that are "off" and vice versa.

Main factors	Yield (kg)	Fruits /tree	CE (kg/cm^2)	Nut (g)
Irrigation treatment				
T_0	6.39	7744.6	0.027	1.042
T_1	4.903	6004.4	0.02	1.025
T_2	7.502	9552.5	0.023	1.001
significance	ns	ns	ns	ns
Year				
2014	5.196	6660.7	0.029	1.211
2015	7.334	8873.4	0.018	0.834
significance	*	ns	**	***

Tab. 3.1 – Effect of irrigation treatments and of year on the following parameters: yield (kg of dry nuts), number of fruits per tree, crop efficiency (CE, yield/trunk cross sectional area), nut dry weight (g). ns not significant ($P > 0.05$). *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$.

4. Conclusions

This study revealed that irrigation had a positive effect on ecophysiological parameters in pistachio trees, even though the quantities of water applied through irrigation, in both years, were below the 50% of the Et_c.

The significant differences of stem water potential found among irrigation treatments, suggest Ψ_{SWP} as an efficient parameter to monitor plant water status, as reported for other deciduous species (Shackel *et al.*, 1997) and as an useful tool to schedule irrigation when low volume of water are applied.

The water relations in both years were dynamic, changing throughout the growing season and were related to phenological stages of nut development. In no water stress conditions (around -1 MPa), occurring during stage 1, the differences between irrigation treatments as concerning assimilation rate and stomatal conductance were small. The low values recorded in this stage were related to an incomplete development of the leaf and thus probably to restricted photosynthetic capacities. As water stress progressed, rainfed trees showed assimilation rate values lower with respect to the irrigated ones and differences were found in the remaining season. Moreover, in 2015 irrigated trees maintained in both stages 2 and 3 maximum assimilation rate values up to 15 μmol m^{-2} s^{-1}; whereas in 2014, at the same stages, resulted a strong decrement of photosynthetic assimilation.

The relationship found between $\Psi_{SWP,}$ A_{max} and g_s below stem water potential values of -1.5 MPa suggested this value as a threshold to indicate the beginning of severe water stress condition in pistachio trees and so it can be used as indicator for irrigation scheduling.

As concerning the productive parameters, no significant effect of irrigation treatments was found on nut yield, buds retention as well as the alternate bearing; probably the amount of water applied in this two-year experiment was too low to arise the positive effect that irrigation has on those parameters.

The here reported results suggested to continue studying over more years the response of pistachio trees to irrigation, aims to vary irrigation schedule improving the water efficiency, especially in an environment characterized by low water availability particularly for agricultural purposes.

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Experiment 2

Evaluation of photosynthetic parameters of pistachio leaf (*Pistacia vera* L.) from A/C$_i$ curves analysis

1. Introduction

The steady-state mechanistic model of C3 photosynthetic carbon assimilation (Farquhar *et al*., 1980) is fundamental to understand how photosynthesis responds to environmental variations. This model has become a common tool to quantify the biochemical processes, underlying the photosynthetic responses of plants in several environmental conditions (Wullschleger, 1993; Long, 1991; von Caemmerer, 2000; Flexas and Medrano 2002a; Medrano *et al*., 2002; Flexas *et al*., 2004; Manter and Kerrigan, 2004). In the last years, both mean temperature and atmospheric CO_2 concentration have increased and are expected to increase more in the next future; thus predicting photosynthetic changes in response to carbon dioxide concentration and temperature is critical for understanding how to manage crop systems and maximize yield (Brennan *et al.*, 2007; Bernacchi *et al.*, 2009). In Farquhar *et al*. model (1980), the biochemical reactions of photosynthesis are considered to be one of two distinct steady states. In the first state, the rate of photosynthesis can be predicted by the properties of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), assuming a saturating supply of substrate (RuBP – ribulose 1,5-bisphosphate). This state is called Rubisco-limited photosynthesis and normally occurs when the CO_2 concentration is low (generally < 200 ppm). The limitation by Rubisco is associated with the low CO_2 concentration, rather than the maximum velocity of Rubisco for carboxylation (V_{cmax}) of the enzyme. In the other state, photosynthetic rates are predicted

assuming that the rate of regeneration of RuBP is limiting and RuBP is used at a constant rate; this is called RuBP regeneration - limited photosynthesis. This condition occurs at higher CO_2 concentration (generally > 200 ppm). A third state occurs, occasionally, when the chloroplast reactions have a higher capacity than the capacity of the leaf to use the products of the chloroplasts primarily, but not exclusively, triose phosphate. This third state is called triose phosphate use (TPU) limitation (Fig. 1.1).
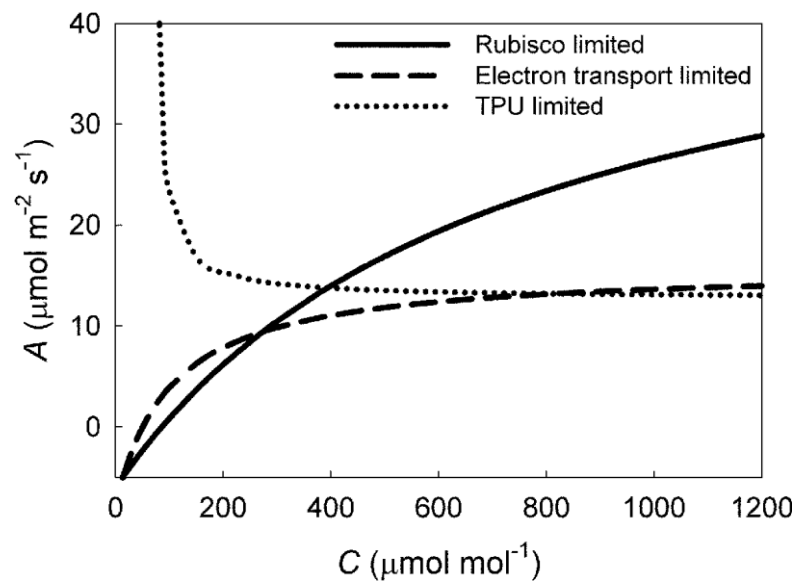
Fig. 1.1 - Representation of photosynthetic response (A) to CO_2 concentration (C) at saturating light, and the three potential biochemical limitations. At low CO_2 concentrations, the rate is limited by Rubisco, then by electron transport, and at very high CO_2 concentrations by triose phosphate utilization (TPU) (from Bernacchi *et al.*, 2009).

When photosynthesis is Rubisco-limited, the response of A to CO_2 concentration can be described by the following equation (1):

$$A = V_{cmax} \left[\frac{C_c - \Gamma *}{C_c + K_c (1 + O/K_o)} \right] - R_d$$

where:

V_{cmax} is the maximum velocity of Rubisco for carboxylation;

C_c is the CO_2 partial pressure at Rubisco;

Γ^* is the photorespiratory compensation point;

R_d is respiratory CO_2 release other than by photorespiration (day respiration) and it is presumed to be primarily mitochondrial respiration;

K_c is the Michaelis constant of Rubisco for carbon dioxide;

K_o is the inhibition constant (usually taken to be the Michaelis constant) of Rubisco for oxygen;

O is the partial pressure of oxygen at Rubisco.

When photosynthesis is limited by RuBP regeneration, the response of A to CO_2 concentration can be described by the following equation (2):

$$A = J\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} - R_d$$

where:

J is the rate of electron transport (at saturated light J is called J_{max});

C_c is the CO_2 partial pressure at Rubisco;

Γ^* is the photorespiratory compensation point;

R_d is respiratory CO_2 release other than by photorespiration (day respiration).

When photosynthesis is limited by TPU, the response of A to CO_2 concentration can be described by the following equation (3):

$$A = 3TPU - R_d$$

where:

TPU is the rate of use of triose phosphates;

R_d is respiratory CO_2 release other than by photorespiration (day respiration).

Several studies have been demonstrating that photosynthetic parameters vary among leaves within a plant, with genus and species, plant functional type and leaf nitrogen content; furthermore they depend on the capacities for the biochemical reactions that regulate gas-exchanges (Wullschleger, 1993; Manter and Kerrigan, 2004; Baldocchi and Amthor, 2001). It is also well known that photosynthetic parameters of tree species vary throughout the season as leaves expand, age, stress and senesce (Dang *et al.*, 1998; Wilson *et al.*, 2000, 2001; Giulias *et al.*, 2002; Nogués and Alegre, 2002; Xu and Baldocchi, 2003). Moreover, a relationship between V_{cmax} and leaf nitrogen content exists and varies among species (Wilson *et al.*, 2000) and canopy position (Meir *et al.*, 2002).

The Mediterranean Basin is characterized by long, hot and dry summers with high daily irradiance and evaporative demand, as well by significant long-term soil water scarcity (Flexas and Medrano, 2002a). In this kind of environment, the amount of precipitation and the rainfall pattern have a strong influence on summer net photosynthesis, while low temperature normally reduces photosynthetic activities in winter months (Harley *et al.*, 1987; Gratani, 1995; Joffre *et al.*, 1999).

Drought probably is the most common stress in Mediterranean area that affects several morphological, physiological and biochemical aspects of the plants (Robichaux, 1984; Chaves *et al.*, 2009; Anjum *et al.*, 2011). The first response of plants to water deficit is to limit leaf transpiration by stomatal closure that causes a decline of leaf intercellular CO_2 concentration (C_i) and, consequently, the photosynthetic activities (Jones, 1985; Flexas and Medrano, 2002b; Grassi and Magnani, 2005). In several studies have been reported the importance of mesophyll conductance and its role in limiting photosynthesis (Grassi and Magnani, 2005; Warren and Adams, 2006). Under severe water stress condition there is a down-regulation of biochemical capacity to assimilate CO_2 that can be observed as a reduction of V_{cmax} (Flexas *et al.,* 2004; Diaz-Espejo *et al.*, 2007). Several are the studies related to the reduction of photosynthetic activities in

environments characterized by water scarcity, mostly on sclerophyll (Xu and Baldocchi, 2003; Gulias *et al.*, 2002; Diaz-Espejo *et al.*, 2007; Flexas *et al.*, 2004; Medrano *et al.*, 2002) and woody species (Wilson *et al.*, 2000, 2001; Xu and Baldocchi, 2003).

At present, numerous studies have been realized on pistachio tree (*Pistacia vera* L.) related to the physiological behaviour in response to abiotic stress (De Palma and Novello, 1998; Goldhamer and Beede, 2004; Goldhamer, 2005; Guerrero *et al.*, 2006; Gijón *et al.*, 2011); on the contrary, there are not data concerning the characterization of photosynthetic parameters of this species. Pistachio is a nut tree species native to Western Asia and Asia Minor where it is still found growing wild (Crane and Iwakiri, 1981; Behboudian *et al.*, 1986; Goldhamer *et al.*, 1987). Although it is considered a drought and saline tolerant species (Crane and Iwakiri, 1981; Behboudian *et al.*, 1986; Goldhamer *et al.*, 1987), several studies have been reported a positive influence of irrigation on CO_2 assimilation rate (De Palma and Novello, 1998; Gijón *et al.*, 2011). In Sicily, where pistachio orchards are mainly located (Barone and Marra, 2004), this species has been traditionally cultivated in dry and marginal areas. But in the last years and in some areas of the island, new irrigated orchards have been developed. We undertook a two-year study on photosynthetic response to CO_2 concentration in pistachio trees. The aim was obtain a quantitative seasonal estimation of photosynthetic parameters in pistachio trees under three different irrigation treatments.

2. Materials and methods

2.1 Site of the research and plant material

The trial was carried out during two years (2014 and 2015) in the same pistachio orchard were the first experiment was done (see above, page 7); the trees used for the trial were also the same. The orchard was managed following the standard cultural practices as commonly recommended to the fanners were adopted for the study.

Water was supplied to the plants by two pressure compensating integral drip-lines per row, with emitters spaced 80 cm along the pipe. The irrigation treatments were as follows:

- T_0: rainfed conditions (control);
- T_1: 50 mm of water supplied (by 1.6 L h^{-1} emitters);
- T_2: 100 mm of water supplied (by 3.5 L h^{-1} emitters).

In 2014 water was distributed in 7 irrigations throughout the growing season from the middle of June to mid-August, while in 2015 water was distributed in 10 times, from the end of June to the middle of August. The total amount of water applied in 2014 was 46.24 mm in T_1 treatment and 101.15 mm in T_2, while in 2015 it was 43.52 mm in treatment T_1 and 95.17 mm in T_2 treatment (as above reported in the first experiment, page 9).

Three blocks, constituted by three adjacent rows each, were replicated for each irrigation treatment. Three trees for each block were selected for their uniformity in the central row for a total of 9 trees per treatment.

2.2 Meteorological Data

Daily climatic data were acquired from a public weather station (SIAS - Servizio Informativo Agrometeorologico Siciliano), positioned next to the experimental site. To characterize the site were considered the following data: maximum and minimum air temperature (°C), rain (mm), maximum and minimum relative humidity (%), wind speed (m/s) and global solar radiation (MJ/mq).

2.3 Plants water status

Water status of the trees was monitored measuring midday steam water potential (Ψ_{SWP}) with a Scholander pressure chamber (PMS Instrument Co., Corvallis – Oregon, USA); measurements were made following the same procedure reported in the first experiment (see page 10). Dates sampling are reported in table 2.1.

2.4 Photosynthetic response to CO_2 concentration (A/C_i curves)

A/C_i response curves (net CO_2 assimilation rate, A, versus calculated internal CO_2 concentrations, C_i) were measured using a portable infrared gas analyzer (CIRAS - 3, PP Systems. Amesbury, Massachusetts, USA) connected to an automatic leaf cuvette (PLC6 (U) PP Systems).

A/C_i curves were made on two sunlight and fully expanded leaves for each irrigation treatment (six leaves total), selected in bearing branchlets and positioned the middle part of the canopy. The leaf chamber of the cuvette covered an exposed leaf area of 2.5 cm^2. During all measurements cuvette conditions were maintained at a constant photosynthetic photon flux density of 1500 µmol m^{-2} s^{-1}, temperature at 27 °C and flow rate at 200 ml min^{-1}; saturating radiation was provided by a LED light unit. A/C_i curves were generated by stepwise decreases starting at CO_2 concentration of 1800 ppm down to 5 ppm at

constant steps (1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200, 100, 50 and 5 ppm); measurements were recorded after equilibration to a steady state.

In table 2.1 were reported the dates were A/C_i curves were done.

2014		2015	
Day	**DAFB**	**Day**	**DAFB**
June 9	47	May 20	23
July 11	80	June 26	60
July 24	93	July 4	78
August 7	107	August 7	102
August 18	118	August 27	122

Tab. 2.1 - Date sampling in 2014 and 2015 and their corresponding days after full bloom (DAFB). During the two-year experiment full bloom was April 24 in 2014, and April 27 in 2015.

The photosynthesis model proposed by Sharkey *et al.* (2007) was used to analyze the A/C_i curves obtained. Data acquired from the curves were included into a spread sheet (www.blackwellpublishing.com/plantsci/pcecalculation) and adjusted manually until each modeled line meets or exceeds all of the data points. From the A/C_i curves analysis was obtained a quantification of important photosynthetic parameters related to leaf physiology:

- maximum carboxylation rate allowed by ribulose 1,5 bisphosphate carboxylase/oxygenase Rubisco (V_{cmax});
- rate of photosynthetic electron transport at saturated light (J_{max});
- rate of triose phosphate use (TPU);
- day respiration rate (R_d);
- mesophyll conductance (g_m).

Values of the photosynthetic parameters were obtained at the measured temperature inside the cuvette and adjusted to 25 °C to facilitate comparisons between measurements.

The accuracy of the photosynthesis model depends on a proper representation of the kinetic properties of Rubisco. The kinetic properties of Rubisco among C3 plants have been shown to be relatively conserved and in this model, a general set of kinetic parameters was used (Tab. 2.2).

	25°C	c	ΔH_a	ΔH_d	ΔS
Parameters used for fitting					
K_c (Pa)	27.238	35.9774	80.99		
K_o (kPa)	16.582	12.3772	23.72		
Γ^* (Pa)	3.743	11.187	24.46		
Parameters used for normalizing					
V_{cmax}	1	26.355	65.33		
J	1	17.71	43.9		
TPU	1	21.46	53.1	201.8	0.65
R_d (μmol m^{-2} s^{-1})	1	18.7145	46.39		
g_m (μmol m^{-2} s^{-1} Pa^{-1})	1	20.01	49.6	437.4	1.4

Tab. 2.2 - The scaling constant (c) and enthalpies of activation (ΔH_a), deactivation (ΔH_d) and entropy (ΔS) describing the temperature responses for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), enzyme kinetic parameters and mesophyll conductance necessary for A/C$_i$ curves analysis over a range of temperature. Values are taken from Bernacchi *et al.* (2001, 2002) and Bernacchi, Pimentel and Long (2003); TPU data are taken from Harley *et al.* (1992). K_c is Michaelis constant of Rubisco for triose phosphate use; R_d is the day respiration and g_m is mesophyll conductance; K_o is the inhibition constant; Γ^* is the photorespiratory compensation point; V_{cmax} is the maximum carboxylation rate allowed by Rubisco; J is the rate of photosynthetic electron transport; TPU is the triose phosphate use; R_d is the day respiration and g_m is mesophyll conductance.

2.5 Statistical analysis

Data were analyzed using Systat 13.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606). ANOVA and Tukey's multiple range test

was performed for comparing the means between treatments; differences were considered statistically significant at $P < 0.05$.

Relationships among parameters were fitted using Sigmaplot 12.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606).

3. Results and discussion

3.1 Meteorological data

Figure 3.1 shows the thermopluviometric patterns in 2014 (a) and 2015 (b). Temperature in both years followed the same trend. The coldest month was January both in 2014 (average 5.2 °C) and 2015 (average 3.5 °C), while the hottest month was August in 2014 (average 34.6 °C) and again August in 2015 (average 35.5 °C). Cumulated precipitations were 421.2 mm in 2014 and 682.6 mm in 2015. In 2015 rainfall was definitely higher than the 30 years mean value reported for the area where orchard is located (approximately 550 mm as reported by Cartabellotta *et al*., 1998). In both years, rain was concentrated during the fall-winter months. In 2014 March was the rainiest month (113.4 mm), while in 2015 it was February (191.2 mm); the driest month was August in 2014 (0 mm) and July in 2015 (6.6 mm).

From April to August effective rainfalls were 57.4 mm in 2014 and 80 mm in 2015 (34.6 mm were recorded only in August). Taking into consideration the irrigation during the same period, the total amount of water received by trees (rainfall + irrigation) in 2014 was 103.6 mm in T_1 and 158.5 in T_2 trees, while it was 123.5 mm in T_1 and 175.2 mm in T_2 plants in 2015. Rainfed trees received through only rainfalls 57.4 mm in 2014 and 80 mm in 2015. The biggest differences between the years were found in August, in fact in 2014 trees received 0 mm (T_0), 4 mm (T_1) and 9 mm (T_2) with respect to 34.6 mm (T_0), 50.9 mm (T_1) and 70.29 mm (T_2) in 2015.
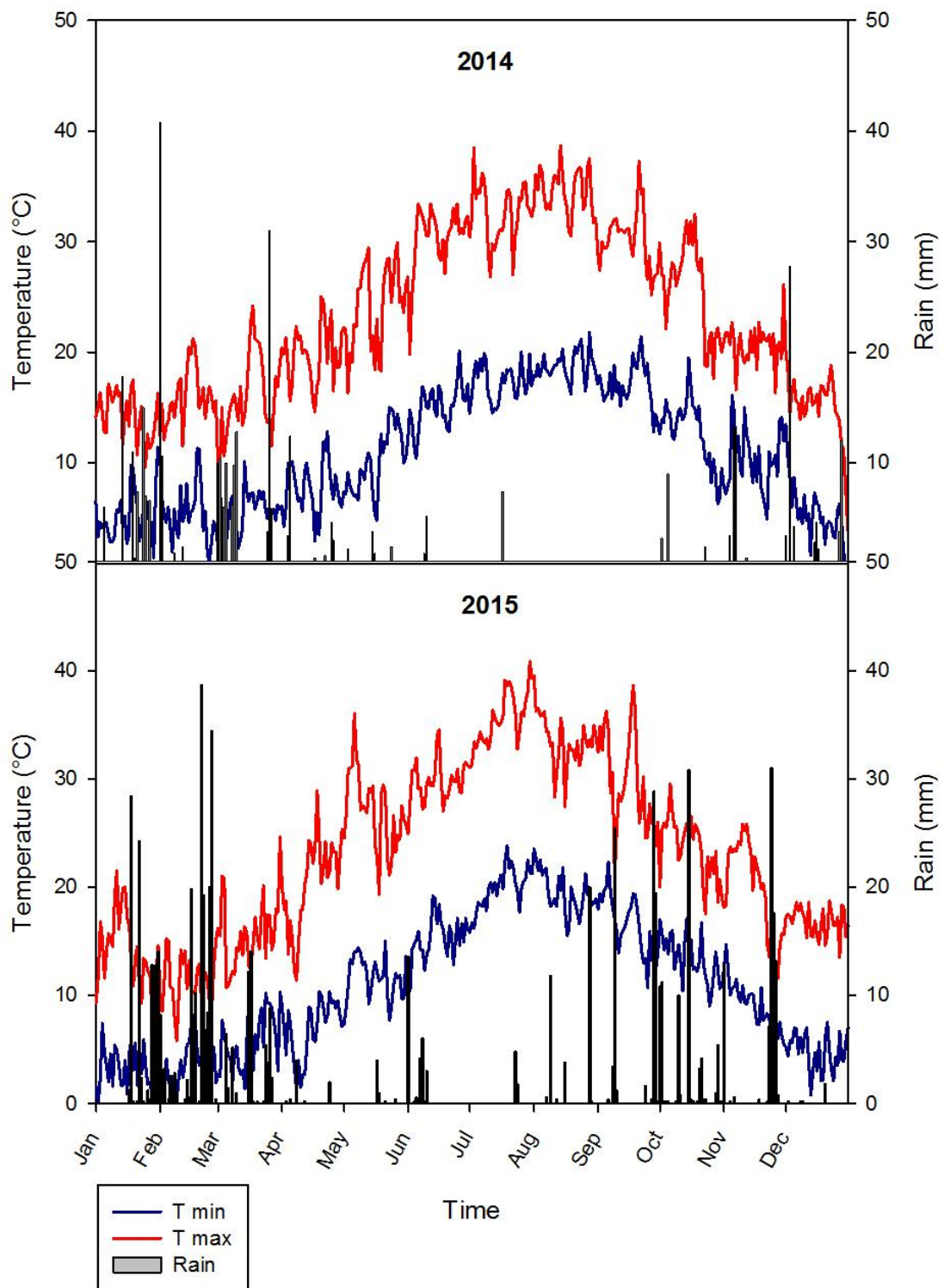
Fig. 3.1 – Seasonal patterns of daily air temperature (red and blue lines for maximum and minimum temperatures respectively) and total rainfall (grey bars) in 2014 (a) and 2015 (b).

3.2 Stem water potential

Figures 3.2 show the patterns of stem water potential (Ψ_{SWP}) during the dates sampling where A/C$_i$ curves were made in 2014 (from June to August) (a) and 2015 (from May to August) (b), as affected by different irrigation treatments. In both years patterns were similar and decreasing in midsummer, as also it has been reported for pistachio tree by Goldhamer (2005), Gijón *et al.* (2009) and Memmi *et al.* (2016).

In details, in 2014 (fig. 3.2 a) at 47 DAFB in all treatments Ψ_{SWP} values were around -1.3 MPa; This values have been reported in several studies in the first stage of pistachio (Guerrero *et al.*, 2006; Gijón *et al.*, 2009). Then the stress level started to increase, specifically in rainfed plants, and significant differences between irrigation treatments, were found. From mid-July to the end of August, no rainfall events happened and water was applied only one day in mid-August; thus as consequence was observed a very strong increment of stress in all treatments. The lowest Ψ_{SWP} values were observed 118 DAFB when all plants were strongly stressed (values ranging from -1.9 to -2.3 MPa).

In 2015 pattern was similar to that in previous year (Fig. 3.2 b). In fact a strong reduction in all treatments was observed 78 DAFB (mid-July), with Ψ_{SWP} values ranged from -1.9 MPa to -2.2 MPa. The lowest Ψ_{SWP} values were recorded at the beginning of August (102 DAFB). At 122 DAFB, after several rainfall and irrigation events, values were less negative in irrigated plants (-1.7 MPa in T_1 and -1.6 MPa in T_2 trees respectively); on the contrary in rainfed treatment no recovery was observed, showing Ψ_{SWP} values that denote high degree of water stress (-2.1 MPa). The re-watering effect observed in irrigated plants probably was due both to the irrigation and rainfall events, whereas only rainfall events (16 mm total) were not sufficient to rehydrate T_0 trees.
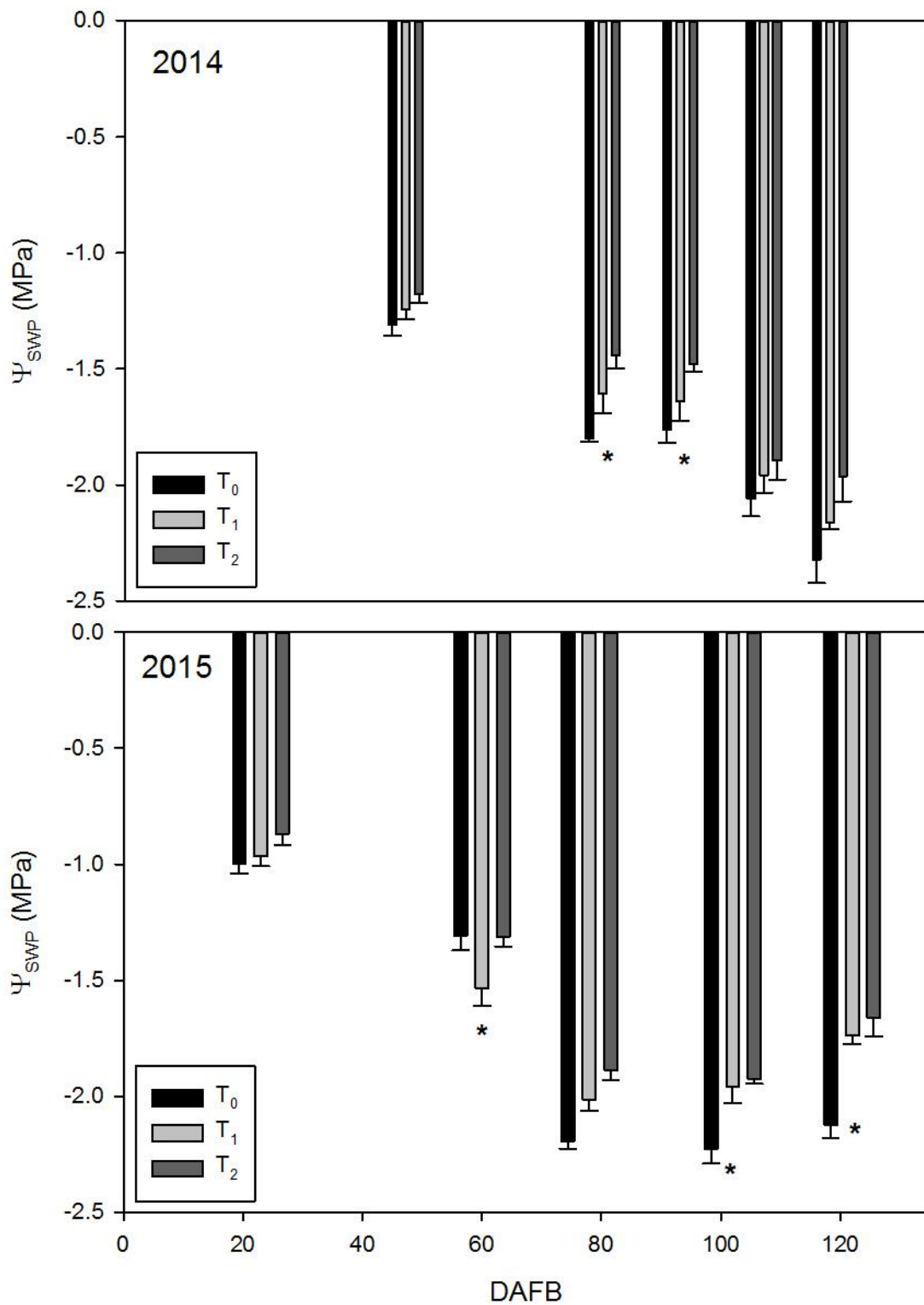
Fig. 3.2 - Midday stem water potential (Ψ_{SWP}, MPa) in pistachio trees during the period where A/C$_i$ curves were done in 2014 (a) and 2015 (b), as affected by irrigation treatments. Values are mean ± S.E. Asterisks denote the dates when significant differences were found among irrigation treatments (P < 0.05).

3.3 Seasonal patterns of photosynthetic parameters

The seasonal pattern of maximum rate of RuBP carboxylation (V_{cmax}) as affected by irrigation treatments in 2014 is showed in fig. 3.3 a.

The trend was decreasing in all treatments throughout the growing season. At the beginning of June (47 DAFB) and until 93 DAFB (July) all treatments showed a mean V_{cmax} value of around 150 µmol m^{-2} s^{-1} that it was nearly stable and not related to plants water status. From 107 DAFB (the beginning of August) a high reduction of V_{cmax} was observed in all treatments, correlated to a decrement of Ψ_{SWP} (around -2 MPa). Later, V_{cmax} continued decreasing, reaching the lowest values of the season. Overall, from June to August was observed a strong reduction of V_{cmax} in rainfed trees (57%) and in T_1 treatments (33%), while T_2 trees showed similar V_{cmax} values (around 100 µmol m^{-2} s^{-1}) during the whole period.

In 2015, from 23 DAFB (mid-May), V_{cmax} values started to increase (fig. 3.3 b), reaching the highest values at 78 DAFB (July); in this phase leaves were completely developed, reaching probably the highest photosynthetic capacities (Lin *et al.*, 1984; Novello, 1998; Vemmos, 1994). Then was observed a decline of V_{cmax}; specifically T_0 trees showed the deepest reduction, while in T_2 trees were still observed high values (around 200 µmol m^{-2} s^{-1}). At the end of August (122 DAFB), also irrigated trees showed a fall of V_{cmax}, probably due to the high water stress reached (-2 MPa).

Overall in 2015, we observed higher carboxylation rates with respect to 2014; these differences, which have also been observed in the previous experiment about the assimilation rate, were probably correlated to the different Ψ_{SWP} values reached by the trees. Specifically in August 2015, irrigated trees showed values ranging from -1.6 to -1.9 MPa, while in 2014 they ranged from -1.9 to -2.3 MPa.

In figures 3.4 a (2014) and b (2015) are reported the seasonal patterns of the maximum rate of electron transport driving regeneration of RuBP (J_{max}), as affected by irrigation treatments. The J_{max} trends were decreasing throughout the season in both years, similarly to the V_{cmax} patterns.

In 2014 (fig. 3.4 a) until 93 DAFB (the end of July) in all treatments high values were recorded, while then was observed a reduction and 118 DAFB (the end of August) J_{max} values ranged from 100 to 150 µmol m^{-2} s^{-1}. Overall, from the beginning of the season to the end, was measured a reduction of 52% in rainfed trees and 41% in T_1 trees; on the contrary in T_2 trees no differences were observed (around 150 µmol m^{-2} s^{-1}). J_{max} pattern in 2015 is shown in fig 3.4 b; the trend was similar to that in previous year. Until 78 DAFB, all treatments showed similar values (around 170 µmol m^{-2} s^{-1}), while 102 DAFB (the beginning of August) values started decreasing until the end of August (122 DAFB).

A positive correlation between V_{cmax} and J_{max} was found and it indicates that the assimilation of CO_2 is regulated in a co-ordinating manner by these two components processes (data not showed).

It is well known that photosynthetic activities are species-specific and depend on the capacities for the biochemical reactions that regulate all the gas-exchange processes. Wullschleger, in a photosynthetic study across 103 C3 species (1993), found that V_{cmax} values ranged from 6 µmol m^{-2} s^{-1} for the coniferous species *Picea abies* to 194 µmol m^{-2} s^{-1} for the agricultural species *Beta vulgaris* and reported a mean of 64 µmol m^{-2} s^{-1} among all the studied species. He also found that J_{max} values ranged from 17 µmol m^{-2} s^{-1} for *Picea abies* to 372 µmol m^{-2} s^{-1} for the desert annual *Malvastrum ratundifolium*; among all species a mean of 134 µmol m^{-2} s^{-1} has reported in the same study. For fruit trees species, Wullschleger reported 37 as average of V_{cmax} and 82 as average of J_{max}.

Our values are definitely higher compared than those reported by Wullschleger; in details we obtained a V_{cmax} mean value of 146 µmol m^{-2} s^{-1} (minimum 71 µmol m^{-2} s^{-1} and maximum 222 µmol m^{-2} s^{-1}), and a J_{max} mean value of 167 µmol m^{-2} s^{-1} (minimum 96 µmol m^{-2} s^{-1} and maximum 235 µmol m^{-2} s^{-1}). The highest values reported in that study were related to desert annuals and perennials species (V_{cmax} mean value of 156 µmol m^{-2} s^{-1} and J_{max} mean value of 306 µmol m^{-2} s^{-1}). The similarity of our data to those about desert species could be so explain the high drought tolerance of pistachio tree.

To clarify the effect of the water status on photosynthetic parameters, V_{cmax} and J_{max} were correlated with stem water potential (figures 3.5 and 3.6). A weak relationship between V_{cmax} and Ψ_{SWP} was found (P<0.001; R^2= 0.39), while for J_{max}, although an upward trend is evident, not a significant correlation was found. As shown in the figures, both parameters started decreasing at Ψ_{SWP} values of around -1.5 MPa, reaching the minimum values related to the lowest Ψ_{SWP} values (around -2.5 MPa); a similar pattern was observed also in the previous experiment related to the photosynthetic assimilation and also in agreement with Memmi *et al*. (2016). Nevertheless we cannot differentiate completely the factors that caused these reductions. The first response of plants to water deficit is to limit leaf transpiration by stomatal closure (Jones, 1985; Flexas and Medrano, 2002b; Grassi and Magnani, 2005); further it has also been reported that there is a down-regulation of biochemical capacity to assimilate CO_2 (Flexas *et al*., 2004; Diaz-Espejo *et al*., 2007). Photosynthetic capacity is closely correlated to nitrogen through the nitrogen-rich carbon-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Wilson *et al*., 1999). Has been reported that nitrogen allocated to Rubisco can vary with leaf age (Poorter and Evans, 1998; Rey and Jarvis, 1998; Wilson *et al*., 2000). Also in pistachio has reported a decreasing of nitrogen amount throughout the season (Weinbaun and Muraoka, 1986; Picchioni *et al*., 1997). Decrements of photosynthetic capacities have been observed by Wilson *et al*. (2000) and Xu *et al*. (2003) in deciduous forest under prolonged summer drought and high temperature and they were related to leaf senescence, water stress and nitrogen amount. Furthermore a strong decreasing of Rubisco activity is commonly reported only under very severe stress; in fact inhibition at mild to moderate state were occasionally reported (Castrillo and Calcagno, 1989; Holaday *et al*., 1992; Medrano *et al*., 1997) and photoinhibition eventually occurs in a very severe stress condition (Flexas and Medrano, 2002b).
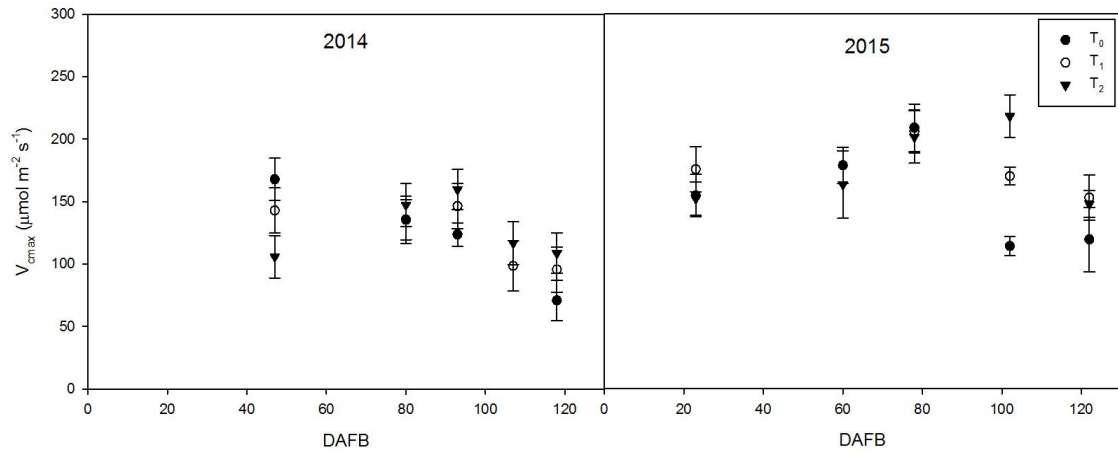
Fig. 3.3 - Seasonal evolution of the maximum rate of RuBP carboxylation (V_{cmax}, μmol m^{-2} s^{-1}) (at 25°C) as affected by irrigation treatments in 2014 (a) and 2015 (b). Closed circles rainfed trees, open circles T_1 treatment and closed triangles T_2 treatment. Values are mean ± S.E.
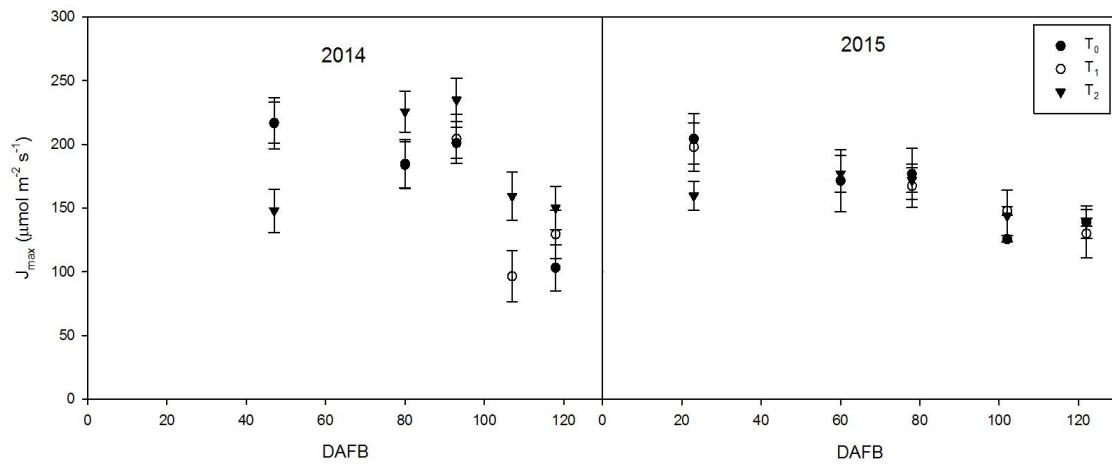
Fig. 3.4 - Seasonal trend of the maximum rate of electron transport driving regeneration of RuBP (J_{max}, μmol m^{-2} s^{-1}) (at 25°C) as affected by irrigation treatments in 2014 (a) and 2015 (b). Closed circles rainfed trees, open circles T_1 treatment and closed triangles T_2 treatment. Values are mean ± S.E.
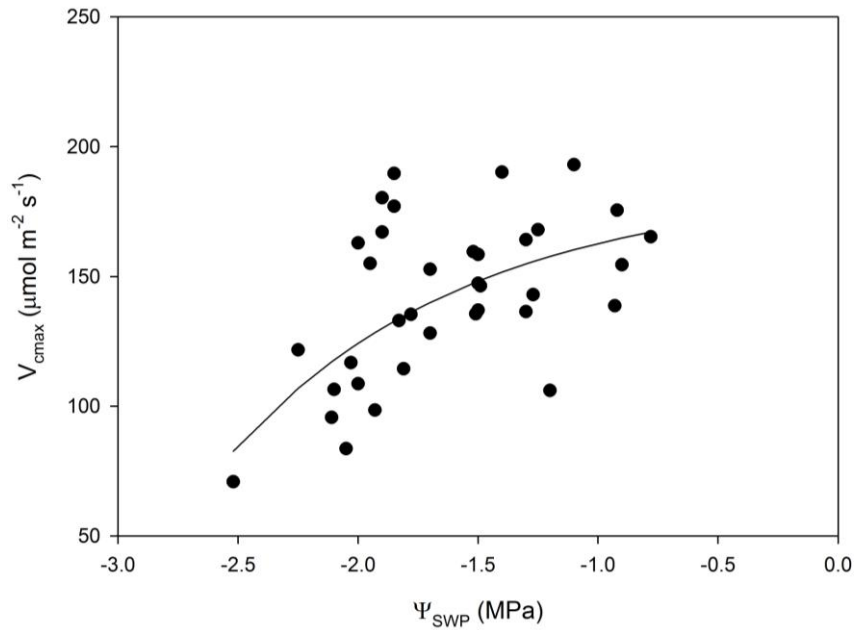
Fig. 3.5 - Relationship between the maximum rate of RuBP carboxylation (V_{cmax}, μmol m^{-2} s^{-1}) and the stem water potential (Ψ_{SWP}, MPa) during all the growing season. Pooled data of 2014 and 2015 were used. The best fit relationship was obtained using an exponential rise to maximum equation y=a+bexp(-x). The parameters are a= 185.1; b= -8.24; R^2=0.39. P$\leq$ 0.0001. Values showed are data points.
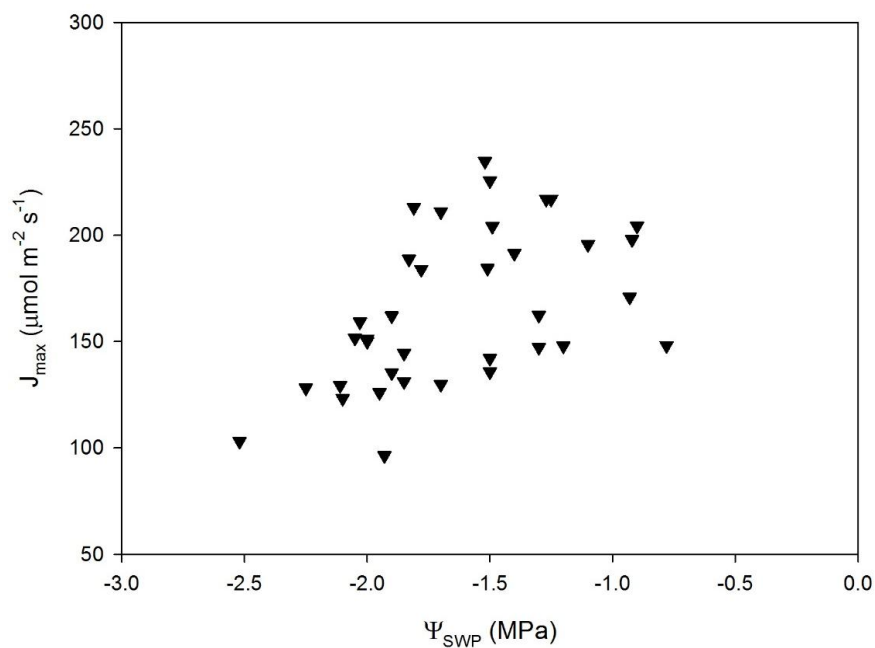
Fig. 3.6 - Relationship between the maximum rate of electron transport driving regeneration of RuBP (J_{max}, μmol m^{-2} s^{-1}) the stem water potential (Ψ_{SWP}, MPa) during all the growing season. Data from 2014 and 2015 were used. Values showed are data points.

From A/C$_i$ curves analyses were also calculated the following parameters: rate of the use of triose phosphates (TPU), daily respiration rate (R$_d$) and mesophyll conductance (g$_m$) (table 3.1). Day respiration decreased among the season both in 2014 and 2015; the maximum values were recorded at the beginning of the season when leaves showed high day respiration rates related to the intensity of the development activities as reported by Marra *et al.* (2009).

TPU rates decreased during the growing season in both years. The mean value measured for pistachio in our experiment (11 μmol m^{-2} s^{-1}) is included in the range of values reported by Wullschleger ranged from 4.9 μmol m^{-2} s^{-1} for the tropical perennial *Tabebuia rosea* to 20.1 μmol m^{-2} s^{-1} for the weedy annual *Xanthium strumarium* (1993).

Concerning the mesophyll conductance, the seasonal patterns were not clear in both years. In 2015 we observed lower values at the end of the growing season compared to the previous dates sampling. This reduction could be related to leaf senescence process as it has been observed in ash and oak trees by Grassi and Magnani (2005). Anyway Flexas *et al.* (2002 b) reported that mesophyll conductance declined under drought stress; furthermore has been reported that decrement of g$_m$ can be related to impaired carbon anydrase in water stress condition (Beadle and Jarvis, 1977; Cornic *et al.*, 1989; Renou *et al.*, 1990; Lal *et al.*, 1996; Roupsard *et al.*, 1996).

	2014					
DAFB	**TPU** (μmol m^{-2} s^{-1})		**R$_d$** (μmol m^{-2} s^{-1})		**g$_m$** (μmol m^{-2} s^{-1} Pa^{-1})	
	mean	S.E	mean	S.E	mean	S.E
47	12.76	±1.9	12.16	±1.89	1.62	±0.07
80	13.6	±1.03	8.03	±0.72	2.27	±0.3
93	15.53	±1.14	10.59	±0.74	3.4	±1.01
107	8.1	±2.5	3.24	±0.98	2.05	±0.1
118	9.52	±0.37	3.63	±0.43	1.89	±1.06
	2015					
	mean	S.E	mean	S.E	mean	S.E
23	12.57	±1.01	13.46	±1.25	1.23	±0.25
60	13.71	±1.28	10.81	±0.94	0.82	±0.07
78	13.98	±0.59	7.39	±0.72	2.37	±1.32
102	10.86	±0.85	3.86	±2.49	1.43	±0.31
122	10.18	±0.46	3.48	±0.8	0.58	±0.08

Tab. 3.1 - Seasonal values of the rate of use of triose phosphates (TPU, μmol m^{-2} s^{-1}), day respiration (R$_d$, μmol m^{-2} s^{-1}) and mesophyll conductance (g$_m$, μmol m^{-2} s^{-1} Pa^{-1}) obtained from A/C$_i$ curves analysis in 2014 and 2015. Values are mean ± S.E.

4. Conclusions

In this first study has been observed a seasonality of V_{cmax} and J_{max} parameters. Although we did not find a significant effect of the irrigation treatments on photosynthetic parameters, interesting relationships between V_{cmax} and J_{max} and water status were found. Particularly has been found a Ψ_{SWP} value, around -1.5 MPa, that could represent the transition point between mild and severe water stress condition, as it has been supposed also in the previous experiment related to the photosynthetic assimilation rate. Nevertheless in this study we cannot differentiate completely the factors that influenced these parameters throughout the growing seasons; under drought stress decrement of photosynthetic capacities are related to several limitations and it is already well known that leaves development stage and leaf nitrogen content have high effect on photosynthetic processes. Also others photosynthetic parameters obtained from A/C_i curve analysis (rates of use of triose phosphates and rate of respiration) decreased during midsummer in both years, while the seasonality of mesophyll conductance was not clear.

The lack of references in bibliography made difficult compare the obtained results and suggested the necessity to continue studying the photosynthetic capacities of pistachio leaves aims to improve knowledges of the biochemical processes and differentiate between the complex regulation of stomatal and non-stomatal limitations. Moreover, understanding how water status can affect photosynthetic capacities may be a useful tool to manage irrigation schedule in pistachio and improve the water efficiency.

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Experiment 3

Chlorophylls content and volatile compounds in pistachio (*Pistacia vera* L.) as affected by different water stress levels

1. Introduction

Pistachio (*Pistacia vera* L., fam. Anacardiaceae) is a nut tree species native to Western Asia and Asia Minor, that can be cultivated in drought and saline conditions (Behboudian *et al.*, 1986). Iran, USA, Turkey and Syria are the world's main producers of pistachio (FAO, 2013). In Italy the pistachio nut production is mainly located in Sicily (Barone and Marra, 2004), where it is mostly cultivated on lava rich soils around Mount Etna in the Eastern part of the Island. Orchards are also located in the central part of Sicily (Caltanissetta and Agrigento areas) (ISTAT, 2014). Few areas in Sicily are interested by pistachio cultivation, so the production is very low compared to that in other countries (i.e. California and Iran) and represents just 1% of the world production (FAO, 2013); however, it is compensated by very high quality (Di Marco, 1987). Sicilian pistachio is locally known as "Green Gold" and it is an important economic product for the region; it is also worldwide appreciated for the intense green colour and for its nutrients content and organoleptic properties (Giuffrida *et al.*, 2006; Gentile *et al.*, 2007). Worldwide, the majority percentage of pistachios are sold roasted with salt or not, and consumed as snacks and confections. During roasting process several thermal and chemical reactions occur, which finally change some aromatic characteristics of the nuts and the overall sensory quality (Saklar *et al.*, 2001; Hojjati *et al.*, 2013); Pumilia *et al.* (2014) reported that the roasting process reduces the chlorophylls (a + b) content because of their heat

susceptibly. For this reason Sicilian pistachio is sold as green product and used as fresh ingredient in cakes, pastries, ham, mortadella and ice-creams and in the confectionary industries (Angelini, 1987).

From the nutritive point of view, the energy value of pistachio nut is similar to that of almond (2332 kJ/100 g); pistachio is rich in carbohydrates and minerals, mainly potassium (1025 mg/100 g) (USDA SR 18, 2005). Fat content is about 50-70% of total nut weight according to variety. The fatty acids include about 25% monounsaturated (mostly oleic acid), while around 7% is represented by polyunsaturated fatty acid (mainly linoleic acid) (Garcia *et al.*, 1992; Kafkas *et al.*, 1995). As vitamin content, the presence of β-carotene (3.2 mg/kg), thiamin (8.7 mg/kg), vitamin E (23.0 mg/kg), riboflavin (1.6 mg/kg) and ascorbic acid (50.0 mg/kg) has been reported (USDA SR 18, 2005). Furthermore pistachio nut has recently ranked among the first fifty food products with the highest antioxidant potential (Halvorsen *et al.*, 2006).

As concerning the volatile composition of nuts terpenes, alcohols, phenols, acids, esters have already been identified in previous studies in different pistachio cultivars, and α-Pinene, Limonene and β-Pinene (terpenes class) have been reported as major constituents (Kendirci and Onogur, 2011; Roitman *et al.*, 2011; Dragull *et al.*, 2010; Hojjati *et al.*, 2013; Carbonell-Barrachina *et al.*, 2015).

Several studies showed that plant pigments play an important role in health (Mayne *et al.*, 1996; Franceschi *et al.*, 1994). Kritchevsky (1999) and Landrum and Bone (2001) had highlighted the potential health benefits of a diet rich in carotenoids due to their role as antioxidants and as agents preventing cardiovascular diseases. The internal kernel colour is a characteristic visual quality parameter in pistachio nuts and the food processing industry prefers intense green kernels (Giuffrida *et al.*, 2006). Giovannini and Condorelli (1958), in the first study concerning the metabolism of the chloroplast pigments of pistachio nuts, reported the presence of chlorophylls a and b, β-carotene and lutein. Moreover Agar *et al.* (1998) reported data on the variation of chlorophylls levels in pistachio varieties of different origin, highlighting the highest

chlorophylls content in Italian samples. Bellomo and Fallico (2007) in their quantitative analysis of pigments composition, depending on the geographic origin, reported that Italian samples have the highest pigments concentration. Moreover, has been reported that cultivar Bianca is also able to maintain higher chlorophylls amount after 12 and 18 months after the harvest (La Russa *et al.*, 2007); it is known that the more is the time after the harvest the more is the decrement of the pigments composition (Giovannini and Condorelli, 1958).

In Mediterranean basin pistachio is mostly cultivated under rainfed condition; in several studies a positive influence of irrigation on yield (Polito and Pinney, 1999; Goldhamer, 2005; Ak and Agackesen, 2006; Gijón *et al.*, 2009), nut quality (as split nuts) and alternate bearing (Kanber *et al.*, 1993; Goldhamer, 1995) has been reported. It is also well known that water status influences several physiological processes in the plants as stomatal opening, photosynthetic carbon assimilation and leaf expansion (Robichaux, 1984). At present, very few are the researches about the influence of trees water status on the quality attributes of pistachio nuts, as concerns the pigments amount and the volatile composition (Di Martino, 2013; Carbonell-Barrachina *et al.*, 2015). Thus, for all the above-mentioned reasons, the aim of this study was to evaluate the effects of different water stress levels reached by trees, on qualitative characteristics of fresh pistachio nuts, cultivar Bianca. Specifically we focused attention on the influence of the irrigation on the chlorophylls composition (a + b), on the volatile compounds and on some yield parameters.

2. Materials and methods

2.1 Experimental site

The trial was carried out during two years (2014 and 2015) in the same pistachio orchard were the first experiment was done (see above, page 7); the trees used for the trial were also the same. The orchard was managed following the standard cultural practices as commonly recommended to the fanners were adopted for the study.

Water was supplied to the plants by two pressure compensating integral drip-lines per row, with emitters spaced 80 cm along the pipe. The irrigation treatments were as follows:

- T_0: rainfed conditions (control);
- T_1: 50 mm of water supplied (by 1.6 L h^{-1} emitters);
- T_2: 100 mm of water supplied (by 3.5 L h^{-1} emitters).

In 2014 water was distributed in 7 irrigations throughout the growing season from the middle of June to mid-August, while in 2015 water was distributed in 10 times, from the end of June to the middle of August. The total amount of water applied in 2014 was 46.24 mm in T_1 treatment and 101.15 mm in T_2, while in 2015 it was 43.52 mm in treatment T_1 and 95.17 mm in T_2 treatment (as above reported in the first experiment, page 9).

Three blocks, constituted by three adjacent rows each, were replicated for each irrigation treatment. Three trees for each block were selected for their uniformity in the central row for a total of 9 trees per treatment.

Daily climatic data were acquired from a public weather station (SIAS - Servizio Informativo Agrometeorologico Siciliano), positioned next to the experimental site. Cumulated precipitations were 421.2 mm in 2014 and 682.6 mm in 2015. In both years rainfalls were concentrated during Fall-Winter months. In 2014 March

was the rainiest month (113.4 mm) while in 2015 it was February (191.2 mm); the driest month was August in 2014 (0 mm) and July in 2015 (6.6 mm).

Taken into consideration the rainfall during the growing season and the irrigation dates, the total amount of water received by plants in 2014 was 103.6 mm in T_1 and 158.5 in T_2 plants, while it was 123.5 mm in T_1 and 175.2 mm in T_2 plants in 2015; rainfed trees received through only rainfalls 57.4 mm in 2014 and 80 mm in 2015.

2.2 Plants water status

Water status of the trees was monitored measuring midday steam water potential (Ψ_{SWP}) with a Scholander pressure chamber (PMS Instrument Co., Corvallis – Oregon, USA); measurements were made following the same procedure reported in the first experiment (see page 10). Dates sampling are reported in table 2.1 (page 11 of the first experiment).

Ψ_{SWP} data were used to calculate the cumulative midday stem water potential (MSWP), following the method reported by Gucci $et\ al.$ (2007):

$$\frac{(MSWPt1 - MSWPt2)\,(t2 - t1)}{2}$$

where:

- MSWPt1 and MSWPt2 are midday stem water potential measured in two consecutive dates;
- t1 and t2 represent the days when measurements were done.

Using obtained results, trees were divided into three groups, and each represents a different level of water stress reached by trees at the harvest time.

The groups are the following:

- Water stress level 0: cumulated MSWP $<$ -130 MPa;
- Water stress level 1: -130 MPa $\geq$ cumulated MSWP $\geq$ -140 MPa;
- Water stress level 2: cumulated MSWP $>$ -140 MPa.

2.3 Nut yield

At the beginning of the growing season, in both years, were randomly selected 2 branchlets per each tree (eighteen branchlets total).

Pistachio nuts were harvested on the 10th of September in 2014 and on the 14th of September in 2015. Collected nuts were brought the same day in the laboratory. Samples were deshelled, peeled and dried in a ventilated oven at 40°C until a stable weight was reached Yield per tree, number of nuts per branchlet were determined. Fresh and dry weight of nuts (in shell) and kernels were measured.

2.4 Chlorophylls content

The kernel chlorophylls content was determined in dry nut samples. Four kernels per each tree (thirty-six samples total), were deprived of violet tegument and then pulverized with a homogenizer (Sterilmixer PbiBrand. Milano – Italy); 0.25 g of powder were posed in 10 ml flasks with 5 ml of N,N-Dimethylformamide aims to extract chlorophylls (Moran, 1982; Porra *et al.*, 1989; Porra, 2002). Flasks were covered with aluminium foil to avoid the light degradation of pigments and then put in the fridge for 72 hours at 4°C.

The chlorophylls content was determined by using a spectrophotometer and employing Moran (1982) equations:

$$\text{Chlorophyll a} = 12.64 \, A_{664} - 2.99 \, A_{647}$$

$$\text{Chlorophyll b} = -5.6 \, A_{664} + 23.26 \, A_{647}$$

$$\text{Chlorophyll total} = 7.04 \, A_{664} + 20.27 \, A_{647}$$

where:

-A_{664} absorbance at 664 nm;

-A_{647} absorbance at 647 nm.

2.5 Volatile composition

The identification of the volatile compounds was performed with a headspace solid-phase Microextration mode (SPME) associated to a gas chromatograph Focus GC (Thermo Fischer) coupled with a mass spectrometer (MS) detector DSQ II (Thermo Fischer). The gas chromatograph and mass spectrometer (GC/MS) was equipped with a Superlcowax column (Supelco) (30 m * 0.25 i.d., 0.25 μm film thickness). Four samples for each tree (almost 4.5 g) (thirty-six samples total) were deprived of violet tegument. Kernels with the internal standard n-esanolo-d13 (Cambridge Isotope Laboratories, Inc. USA) at a concentration of 4.52 ppm (μg*mL^{-1}) were posed in vials with a PTFE cap (Polytetrafluoroethylene). Vials were covered with aluminum foil to avoid the light degradation and posed in the heater at 50°C for all the night. The day after each vial was posed in hot water at 50°C for two hours with a fiber. Aims to analyze the volatile composition of pistachio was used the fiber DVB/CAR/PDMS 50/30 μm (Supelco, Bellefonte PA) that has been reported as the best solution to extract the highest number of compounds in pistachio (Acena *et al.*, 2011). Then the absorption was performed by headspace mode. After two hours the fiber was introduced in the Gas Chromatograph. The fiber was maintained for 2 minutes at 40°C and then for 22 minutes at 220°C.

Following the instrumental parameters of GS/MS:

-Sink temperature 250°C;

-Full Scan;

-Mass range 35-350 dalton;

-Absorption time 20 minutes (1 min splitless);

-Column flow 1 ml/min;

-Transfer line 280°C;

-Ionization EI (+).

Identifications were based on the comparison of the obtained spectra with those of the Wiley7 and NIST MS Search 2005 mass spectral libraries. Quantification was carried out calculating the area of peak of each component, using the method ICIS of Xcalibur V 1.4 software (Thermo Electron) and following the relation:

$$A_x : Q_x = A_s : Q_s$$

where:

A_x: area of the chromatographic peak of the unknown component X;

Q_x: concentration of unknown component X;

A_s: area of the chromatographic peak of the internal standard;

Q_s : concentration of the internal standard (4.52 ppm).

All the analyses were done in collaboration with the Department of Biological Chemical and Pharmaceutical Science and Technology (University of Palermo).

2.6 Statistical analysis

Data were examined by ANOVA using Systat 13.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606). Differences between means were analyzed using the Tukey's test with significance at $P < 0.05$. Curves were

fitted using Sigmaplot 12.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606).

3. Results and discussions

3.1 Chlorophylls content

The cumulated water stress level reached at the harvest time had a positive and significant influence on the chlorophylls content (Tab. 3.1). Differences between groups were found; the less stressed trees (level 0) showed higher values of chlorophyll a, b and total, compared with the more stressed trees (level 2). A positive and significant correlation ($R^2 = 0.99$) was found between levels of water stress and the total amount of chlorophylls (Fig. 3.1).

Unfortunately, few researches have been done about the influence of trees water status on quality characteristics and on chlorophylls content of pistachio nuts. Our findings are in agreement with results of Di Martino (2011), that found a positive and significant correlation between the quantity of water applied and the chlorophylls content (a + b) in pistachio nuts cultivar Bianca. The total amounts measured in our experiment in both years were higher. Specifically in rainfed trees Di Martino reported as total chlorophyll amount values around 13 mg/100 g (36% less compared our results in more stressed trees, level 2), while in irrigated trees values were around 20 mg/100 g (43% less compared results obtained in less stressed trees, level 0).

On the contrary, in another study reached out on pistachio nuts cultivar Kerman under irrigation, Carbonell-Barrachina *et al.* (2015) found negligible results.

Main factors	Cumulated MSWP (MPa)		Chl a (mg/100g)		Chl b (mg/100g)		Chl tot (mg/100g)	
Stress level								
0	-152.07	a	10.061	a	12.841	a	21.028	a
1	-135.68	b	11.725	ab	16.102	ab	27.682	ab
2	-124.88	b	14.727	b	20.925	b	35.507	b
significance	***		*		*		*	
Year								
2014	-135		12.055		17.582		29.637	
2015	-139		12.286		15.663		26.508	
significance	ns		ns		ns		ns	

Tab. 3.1 Effect of water stress levels and year of samples collection on cumulated midday stem water potential (MSWP, MPa) and on chlorophylls content (a + b) (mg/100g). Chl a= chlorophyll a, chl b= chlorophyll b and chl tot= chlorophyll total. Values in the same column followed by the same letter were not significantly different (Tukey's test P< 0.05); ns not significant (P > 0.05) *P< 0.05 **P< 0.01 ***P< 0.001.
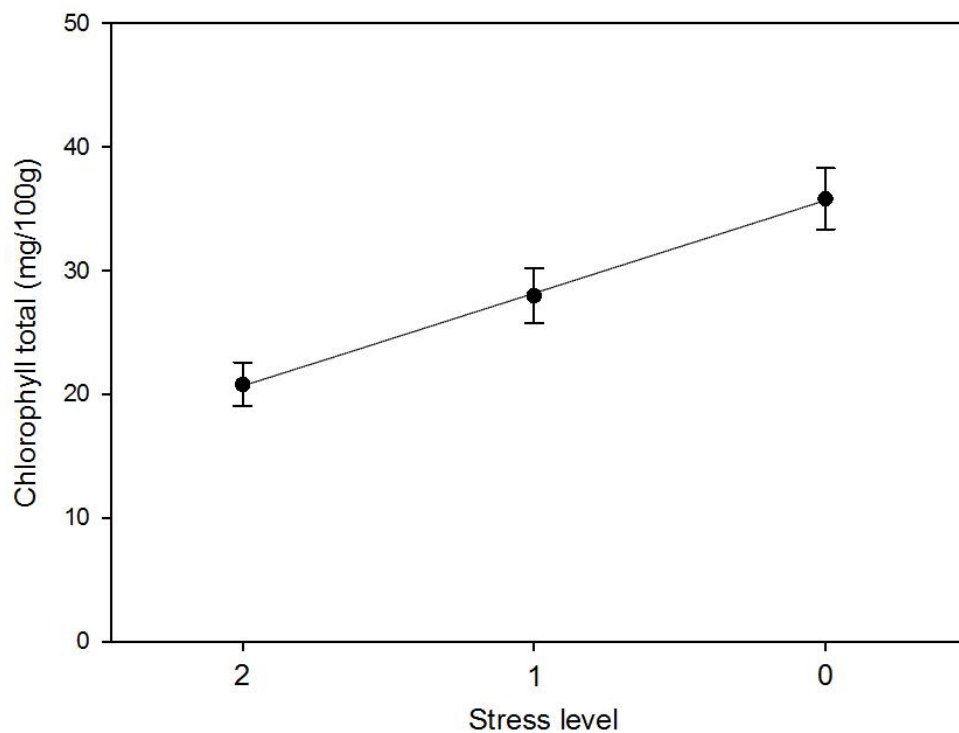
Fig. 3.1 - Relationship between the level of water stress and the total amount of chlorophylls (a + b) (mg/100g). The best fit relationship was obtained using a linear function f = y0+a*x. The parameters are y0= 20.698 a= 7.508 (R^2= 0.99) (P< 0.05). Values are mean ± S.E.

3.2 Volatile composition

The gas chromatograph and mass spectrometer analysis of nuts identified seventeen volatile compounds and for each volatile compound are also reported the respective descriptor (Tab 3.3).

The six most abundant compounds were: α-Pinene (~ 31.41 μg/g), 1-Hexanol (~ 5.43 μg/g), 1H-Pyrrole, 1-methyl (~ 4.94 μg/g), Limonene (~ 2.95 μg/g), 2-Carene (~ 2.43 μg/g) and 1-Nonanol (~ 2.26 μg/g). The volatile profile of nuts was dominated by terpenes and alcohols, also pyrroles, esters and hydrocarbons were found. Terpenes was the predominant class detected (seven volatile compounds on seventeen total).

Our results are supported by previous qualitative and quantitative analysis reached out by other authors, even though there are some differences in samples used (e.g mostly roasted nuts and others cultivars used). In several studies α-Pinene was reported as the main volatile component in essential oil from hull and leaves of *Pistacia vera* L. (Doru *et al.*, 2003; Ozel *et al.*, 2004; Tsokou *et al.*, 2007). In cultivar Kerman Dragull *et al.* (2012) reported Limonene as the main volatile compounds in fruits and leaves, and Roitman *et al.* (2011) found Limonene and β-Myrcene as the most abundant compounds in nuts. Similar results were reported by Kendirci and Onogur (2011) that found α-Pinene, β-Myrcene, Limonene and 1H-Pyrrole, 1-methyl as major components in fresh nuts of four Turkish varieties (Uzun, Kirmizi, Halebi and Siirt ve Ohadi).

Hojjati *et al.* (2013) found α-Pinene and Limonene as the major compounds in fresh and roasted pistachio nuts in three different Iranian varieties of *P. vera* (Ahmad Aghaei, Akbari and Kaleghouchi). Specifically they reported in fresh nuts α-Pinene content of 1.68 μg/g, far and away lower than our results (31.4 μg/g); while the mean amount of Limonene reported in the same study was 1.17 μg/g that is slightly lower than what we found (2.95 μg/g).

Furthermore the major presence of terpenes components, specifically α-Pinene and β-Pinene, have been reported also in fresh nuts of *Pistacia terebinthus*

(Gogus *et al.*, 2011), highlighting that probably it is a characteristic related to *Pistacia* species.

ANOVA test revealed a significant effect of level of water stress in only three of the seventeen volatile compounds found (1-Hexanol, α-Pinene and β-Myrcene). In less stressed plants (level 0) was observed a significantly higher amount compared to plants of level 2 (tab. 3.3); in details the biggest differences were found in α-Pinene that causes the pine and turpentine aroma (around 35 μg/g in level 0 compared to 26.2 μg/g in level 2) and in β-Myrcene (1.4 μg/g in level 0 compared to 0.6 μg/g in level 2) that causes the balsamic and spice aroma. Moreover we noticed that in less stressed trees the amount of terpenes was always higher compared to more stressed trees.

	Level 2		Level 1		Level 0		
Volatile compounds							**Descriptor**
Alcohols							
Ethanol	0.946	ns	0.325		1.322		sweet
1-Pentanol	0.109	ns	0.095		0.326		sweet
1-Hexanol	6.054	b	3.953	a	6.292	b	resin, flower, green
1-Heptanol	0.095	ns	0.074		0.223		mushroom
1-Nonanol	1.573	ns	2.780		2.427		fat, green
Esters							
2,3-Butanediol, [S-(R*,R*)]	0.031	ns	0.054		0.054		fruit, onion
2,3-Butanediol, [R-(R*,R*)]	0.109	ns	0.061		0.081		fruit, onion
Terpenes							
α-Pinene	26.178	a	33.066	ab	34.998	b	pine, turpentine
Camphene	0.685	ns	0.698		0.786		vanilla
β-Pinene	0.529	ns	0.536		0.746		pine, resin, turpentine
β-Myrcene	0.624	a	0.787	ab	1.383	b	balsamic, must, spice
3-Carene	0.325	ns	0.413		0.563		lemon, resin
p-Cymene	0.264	ns	0.332		0.427		solvent, citrus
Limonene	2.807	ns	2.773		3.281		citrus, mint
2-Carene	1.790	ns	2.502		2.997		sweet, pine, cedar
Hydrocarbons							
Undecane	0.251	ns	0.353		0.515		alkane
Pyrroles							
1H-Pyrrole, 1-methyl	5.349	ns	5.438		4.034		alkane

Tab. 3.3 - Volatile compounds (µg/g) of pistachio nuts as affected by different levels of water stress and description of each aromatic compound found. Aim to find the descriptors were used Flavornet and NIST (National Institute of Standards & Technology). Values in the same row followed by the same letter were not significantly different (Tukey's test $P < 0.05$), ns: not significant ($P > 0.05$).

3.3 Nut yield

In table 3.4 are reported the qualitative parameters measured, in terms on number of fruits per branchlet, fresh and dry weight of nut (fruit in shell) and kernel, as affected by the level of water stress. In 2015 was observed a significant higher number of nuts per branchlet than the previous year (61%); at that higher productivity per branchlet corresponded a slightly but significant decrement of fresh and dry weight of both nut and kernel. Whereas no significant differences were found between water stress levels (tab. 3.4). Our results confirm those reported by Carbonell-Barrachina *et al.* (2015) that evidenced negligible results about the effects of irrigation on the weight of kernels cultivar Kerman. Furthermore has been reported that in pistachio trees irrigation can positive influence the yield in terms of an increase of the number of the nuts rather than kernel dry matter accumulation (Goldhamer *et al.*, 1984; Monastra *et al.*, 1997; Gijón *et al.*, 2009).

Main factors	Nuts/ branchlet (n°)	Nut fresh (g)	Kernel fresh (g)	Nut dry (g)	Kernel dry (g)
Stress level					
0	38.529	2.074	0.789	0.859	0.467
1	49.727	1.959	0.768	0.829	0.445
2	46.477	1.967	0.746	0.800	0.438
significance	ns	ns	ns	ns	ns
Year					
2014	28.278	2.468	0.844	1.211	0.489
2015	61.544	1.532	0.692	0.825	0.412
significance	***	***	***	***	***

Tab. 3.3 - Effect of water stress levels and the year of samples collection on following biometric parameters: numbers of fruits per branchlet, fresh weight (F.W.) and dry weight (D.W.) of in-shell fruit and kernel. ns not significant (P > 0.05) *P< 0.05 **P< 0.01 ***P< 0.001.

4. Conclusions

This study revealed that irrigation had a positive effect on the quality traits taken into consideration in this research.

The concentration of pigments chlorophyll a and b, that represents an important eye appeal characteristic, has been influenced by the water stress level reached by the trees.

Seventeen volatile compounds were found, including terpenes, alcohols, esters and only one hydrocarbon and one pyrrol; on them irrigation had always a weak but positive effect. In details, on three of them (α-Pinene, β-Myrcene and 1-Hexanol) in less stressed plants was found a significant higher amount. So irrigation improved the characteristic sensory attributes that cause turpentine, resin, green and balsamic aroma in pistachio nuts. Due to the scarcity of literature found it was difficult comparing our results with others, also because of different kind of samples (e.g. mostly roasted), varieties and methods of extraction used. However the compounds we found in this study are supported by other researches that reported as main volatile components terpenes (Kendirci and Onogur, 2011; Roitman *et al*., 2011; Dragull *et al.*, 2012; Hojjati *et al.*, 2013; Carbonell-Barrachina *et al.*, 2015).

This first investigation on Sicilian pistachio proves that irrigation contributes to an increase of some qualitative characteristics, but the above-mentioned results suggest the necessity to continue studying over more years the effect of water status on qualitative parameters aims to capture the true behaviour of the species.

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Experiment 4

Seasonal changes of carbohydrates content in different organs of walnut trees (*Juglans regia* L.)

1. Introduction

Plants harvest the energy of sunlight by converting light energy to chemical energy. The non-structural carbohydrates (NSCs), synthesized by the Calvin cycle are then converted into storage forms of energy and carbon, and accumulated as resources to be used to support future growth and metabolism (Chapin *et al*., 1990; Richardson *et al*., 2013). The NSCs pool is the sum of soluble sugars, mainly sucrose, plus starch. On the basis of the standard conceptual model, this pool is depleted when demand exceeds supply, for example when requirements for metabolism and growth are high or when photo-assimilates are limited by environmental conditions. On the contrary, the NSCs pool is recharged when the supply exceeds demand, for example when environmental conditions consent high rates of photosynthesis, or when metabolism and growth requirements are low (Chapin *et al*., 1990; Grulke *et al*., 2001; Gleason and Ares, 2004). The dynamics of NSCs are considered indicators of carbon source–sink relationships (Gough *et al*., 2009). It is well known that carbon reserves play an important role in perennial plants, in particular deciduous species, by supplying the required energy for the emergence and growth of new plant organs at the beginning of the growing season (Myers and Kitajima, 2007; Naschitz *et al.*, 2010). Moreover carbohydrates are responsible for most of long distance energy transfers and long term storage of energy.

Starch is the most common reserve carbohydrates in plants, although other carbohydrate, such as hemicellulose and glucans, have also been shown to be mobilized and utilized as sources of energy (Hoch, 2007).

The metabolism of NSCs in perennial plants, like walnut (*Juglans regia* L.), is essential to many aspects of winter biology including frost resistance, survival throughout the dormancy period and the breaking of dormancy (Bohhomme *et al.*, 2005; Charrier *et al.*, 2015). Perennial plants, to survive to dormancy and allow for flowering, need to acquire and store a sufficient supply of carbohydrates, often in the form of starch (Bazot *et al.*, 2013; Menzel *et al.*, 2006). During dormancy, when photosynthesis is limited, trees depend only on stored non-structural carbohydrates to maintain basic metabolic functions, produce defensive compounds and retain cell turgor (Bohhomme *et al.*, 2005; McDowell *et al.*, 2008; Sevanto, 2014; Sperling *et al.*, 2015). In spring, generally before bud break, carbohydrates reserves are rapidly depleted in shoots and roots. In fact an efficient mobilization of stored reserve is essential for bud break and initiation of growth (Rinne *et al.*, 1994; Witt and Sauter, 1994; Simões *et al.*, 2014; Hartmann and Trumbore, 2016). In trees after reaching a minimum before bud break phase, most tissues usually begin to accumulate reserves immediately later (Loescher *et al.*, 1990). Sometimes accumulation is interrupted during the period of fruit ripening (Roper *et al.*, 1988) or slowed down because demand exceeds supply. Then, before leaf abscission, total carbohydrates reserves start increasing and during the winter they remain unchanged or slowly decrease, after which the cycle repeats.

So the vegetative life of any plant can be described as a continuous balance in acquiring, transferring and storing energy that is necessary to grow, reproduce, and protect themselves from environmental abiotic and biotic stress (Zwieniecki and Lampinen, 2015). Temperature is assumed to be the main regulatory signal that determines this progression (Heide, 2008, 2011), so can it effect on the timing of bud break as reported by Alves *et al.* (2004, 2007), Bonhomme *et al.* (2010), Charrier *et al.* (2015) and Zwieniecki *et al.* (2015). The change in global

climate suggests an increase in Mediterranean and temperate areas of diurnal temperature variation (Chaves *et al.*, 2009; Field *et al*., 2014) and therefore implications for trees winter biology (Ameglio *et al*., 2000; Perez *et al*., 2008). It is already well known that yield, as well tree growth, are strongly related to a complex set of interactions involving the genotype, the physiological and developmental processes and their relations with the environment. The understanding of carbohydrates balance in trees responding to environmental conditions may be of key importance to yield predictions, determination of plant stress level and phenology (Zwieniecki and Lampinen, 2015).

So the aim of the present work is to collect preliminary data and quantify the seasonal carbohydrates (soluble sugars and starch) budget in several compartments of walnut trees (*Juglans regia* L.) depending of the phenological stage. Particularly we focused attention on bud-break phases to better understand NSCs mobilization until the harvest time.

2. Material and methods

2.1 Experimental site and plant materials

The experiment was conducted in an orchard owned by University of California, Davis (38°32'16 N, 121°46'32 W; 16 m altitude) on twenty five-year old walnut trees (*Juglans regia* L., cv Chandler on Eastern Black walnut – *J. nigra* L. rootstock). Samples were collected from randomly selected trees (n= 5) and from several organs. Using a drill were collected samples of rootstock (portion above the ground), stem and limb; were collected also samples of roots and branchlets. The branchlets samples were divided into basal and apical part, separating wood from bark (eight samples per each tree, forty samples total). Analysis covered several phenological stages, from the bud dormancy (middle of March) to the harvest time (October). In particular, samples were collected every 10 days during bud dormancy and blooming phases, aimed to follow all the different stages of buds development; then samples were collected once every month (tab. 2.1). Samples were collected always at the same time, around 10 a.m, to prevent diurnal variation of soluble sugar content (Allen *et al*., 2002; Zwieniecki *et al.*, 2015).

The analyses were made in the laboratory of Plant and Environment Sciences of University of California, Davis, under the supervisor of Professor Maciej A. Zwieniecki.

Date sampling	Phenological stages
16 March	Bud dormancy
29 March	Male bloom starting
7 April	Bud-break - First leaves out – male bloom
15 April	Leaves growing
26 April	Female bloom – expanded leaves
11 May	External walnut size growing – fully expanded leaves
10 June	Internal shell and kernel development increment
11 July	Internal shell and kernel development increment
12 August	Internal shell and kernel development increment
7 September	Hulls split
7 October	Harvest time – leaves senescence beginning

Tab. 2.1 – Date sampling and their corresponding phenological stages.

2.2. Determination of non-structural carbohydrates

Samples were dried in the oven at 70°C for 48 hours, then grinded into a fine homogeneous powder using ball mill and weighed almost 25 mg (± 2 mg) of tissue in centrifuge tube. Soluble sugars were extracted by incubating the samples, diluted with ultrapure water (UP), for 15 minutes at 70 °C and centrifuging them for 10 min at 15000 RPM. 50 µL of supernatant was then diluted in new centrifuge tubes with 1000 µL of UP water; soluble sugars were quantified using anthrone reagent (0.1 % (m/v) in 98 % sulfuric acid) by reading absorbance at 620 nm (Levya *et al*., 2008) with a spectrophotometer (Multiscan^{TM} GO microplate, Thermo Fisher Scientific – MA, USA) and using a predetermined standard curve.

Pellet was kept for starch analysis. Starch concentration was measured using a Sigma enzymatic method kit (Sigma, Aldrich - USA). Pellets were washed with ethanol and then with water; 500 µl of acetate buffer (pH = 5.5) (0.5 M Na-

acetate), 0.7 U of amylase and 7 U of amyloglucosidase were then added to samples, and incubated in the oven at 37 °C for 4 hours aims to enzymatic digestion. After, 50 µL of supernatant was diluted in new centrifuge tubes with 1000 µL of UP water; starch was quantified using anthrone reagent (0.1 % (m/v) in 98 % sulfuric acid) by reading absorbance at 620 nm at the spectrophotometer, and using a predetermined standard curve.

2.3 Statistical analysis

Using Systat 13.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606) data were examined. Data were fitted using Sigmaplot 12.0 (Systat Software, Inc. 225 W Washington St., Suite 425 - Chicago, IL 60606).

3. Results and discussions

The dynamics of carbohydrates concentration (soluble sugars + starch) varied significantly during the season and the patterns of variation were different among sampled organs (figures from 3.1 to 3.8).

Storage starch was hydrolysed during the last period of the winter dormancy, before being used for bud break; a very strong reduction of starch concentration was observed in all trees compartments and therefore was measured a consequent increase of soluble sugar concentration. In detail, in roots starch concentration decreased by 85% (from 120 to 20 mg/g of dry weight) (at a later time consequently soluble sugar concentration increased, from 80 to 130 mg/g DW) (fig. 3.1), as well in the woody part of the apical branchlets (from 140 to 40 mg/g DW) (fig. 3.8). In these two sampled organs was observed the highest initial NSCs concentration with respect to the rest of the organs analysed in this experiment. It has been reported in woody species that these compartments probably represent the main storage organs of non-structural carbohydrates (Loescher *et al*., 1990). Furthermore the rapid depletion of carbohydrate reserves, that was rapidly observed in roots, suggested an efficient mobilization of stored reserve necessary for bud break and initiation of growth, as it has been reported by Rinne *et al.* (1994), Witt and Sauter (1994), Simões *et al.* (2014) and Hartmann and Trumbore (2016).

Overall in storage organs, root, rootstock and stem (figures from 3.1 to 3.3) was observed similar patterns of soluble sugars amount, showing a reduction after bud break followed by an increase in the early summer. Same decrease was noticed in other species as *Quercus* twigs by Hoch *et al*. (2003) and in oak by Bazot *et al*. (2013).
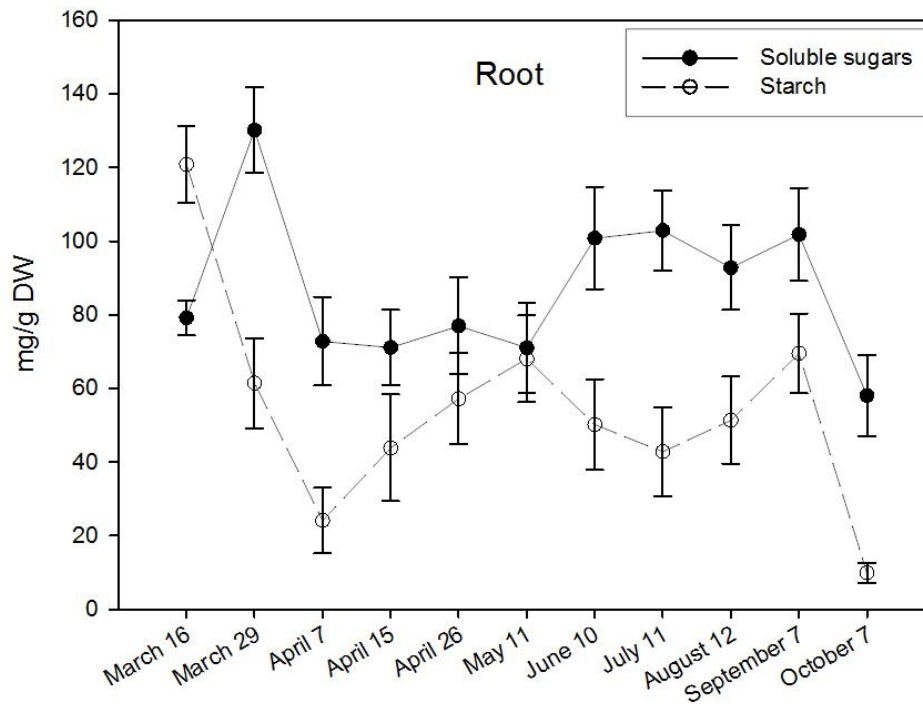
Fig. 3.1 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in the roots throughout the growing season from dormancy to harvest time (March – October).
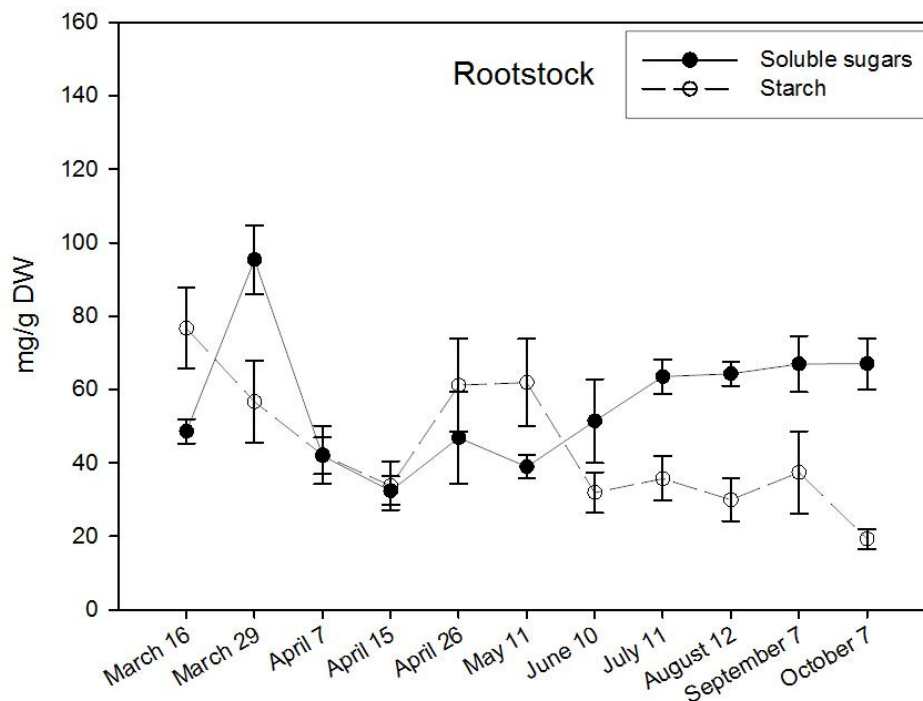
Fig. 3.2 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in the rootstock throughout the growing season from dormancy to harvest time (March – October).
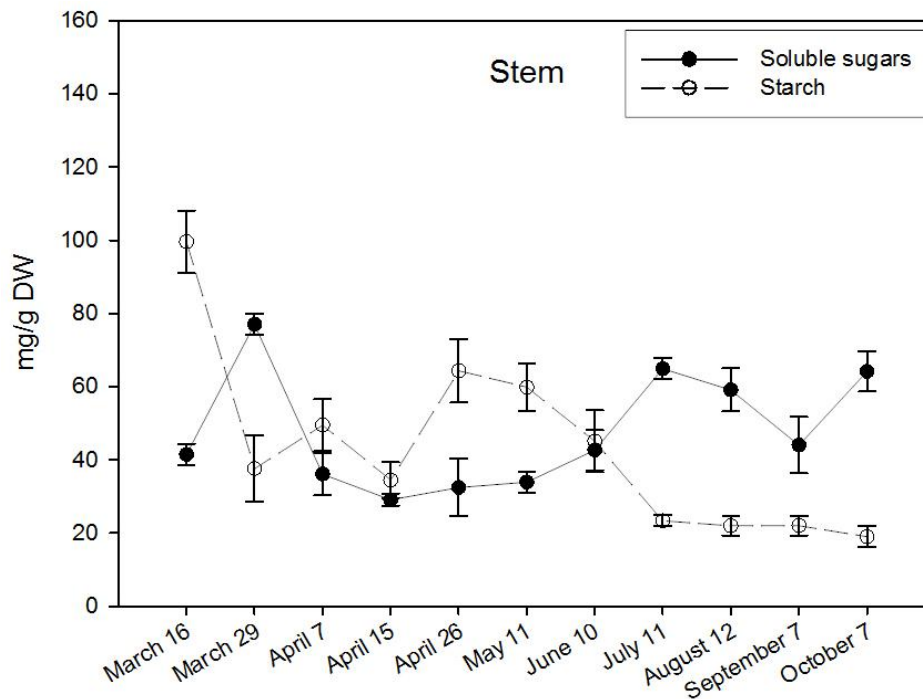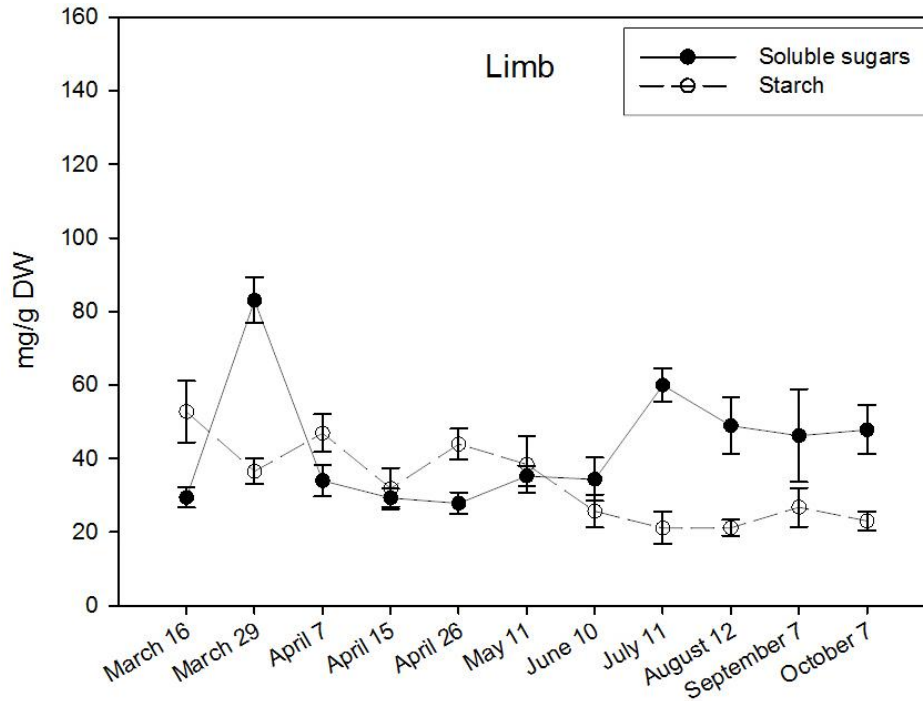
Fig. 3.3 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in the stem throughout the growing season from dormancy to harvest time (March – October).

It is well known that bud break requires carbon supply for metabolic reactivation and leaf primordial growth (Loescher *et al.*, 1990; Gordon and Dejong, 2007; Bonhomme *et al.*, 2010). The very high decrease of the stored starch concentration, that we observed in all organs before bud breaking (the beginning of April), corresponded to the degradation enzyme activity in parenchyma cells as has been reported by Alves *et al.* (2007) and by Rubio *et al.* (2014). It has already been showed that at the time of bud break and growth of new leaves during spring, starch is largely mobilized in several species as *Populus canadensis* (Sauter and van Cleve, 1994), in *Pistacia vera* L (Spann *et al.*, 2008) and in forest trees (Hock *et al.*, 2003). In walnut (*Juglans regia* L.) new stems, flowers and leaves, when are not yet autotrophic, depend upon stored and mobilized reserves for their development (Witt and Sauter, 1994; Laiconte *et al.*, 2004; Bonhomme *et al.*, 2010; Bazot *et al.*, 2013; Dietze *et al.*, 2014), and same

observations have been previously made on the same species by Tixier *et al*. (in press).

In the middle of April leaves started expanding and reached the maximum size in mid-May; so leaves switched from heterotrophic to autotrophic status, started producing photoassimilates. Therefore in this phase in the bark section of the apical and basal parts of the branchlets was observed a rapid increase of soluble sugars concentration (figures 3.5 and 3.7); on the contrary in the woody portion of the same organs the concentration was nearly constant (figures 3.6 and 3.8). The highest amount of soluble sugars in these sampled organs was reached in mid-August; then decreased during the last phase of internal kernel growth highlighting the strong demand of energy aims to complete the fruits development. Overall in these tissues the amount of sugar measured was certainly higher (ranging from 60 to 120 mg/g DW) than to the storage organs (around 60 mg/g DW) (figures 3.3, 3.4 and 3.5), except in roots (ranging from 80 to 100 mg/g DW after bud break). During the period of fruit ripening the amount of starch in branchlets was unvaried indicating probably the necessity to give continuous energy to the fruits development, as reported by Roper *et al.* (1988). It is well known that fruits generally become the dominant sinks during reproductive development, particularly for adjacent and other nearby leaves whereas the root and shoot apices are usually the major sinks during vegetative growth. Anyway, the fact that in branchlets carbohydrates did not reach a complete depletion suggests that reserves were non-limiting as previously reported by Bazot *et al*. (2013). In roots we observed the highest depletion with respect to other organs. Samples were collected until October so is not possible affirm that probably reserve deposition in roots started later in the season aims to survive dormancy and allow for flowering.
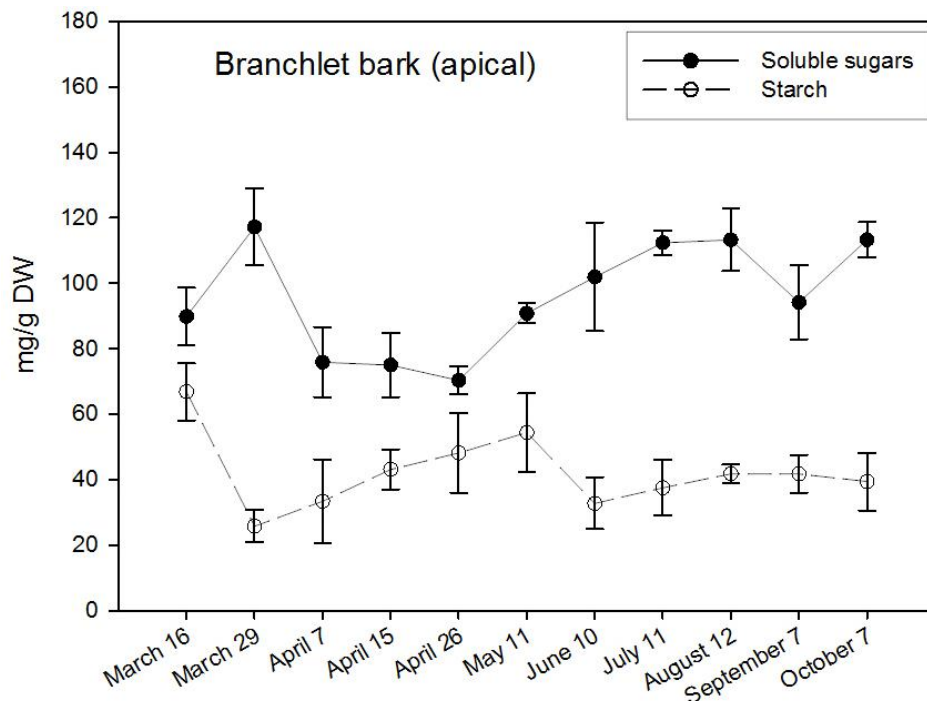
Fig. 3.4 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in the limb throughout the growing season from dormancy to harvest time (March – October).
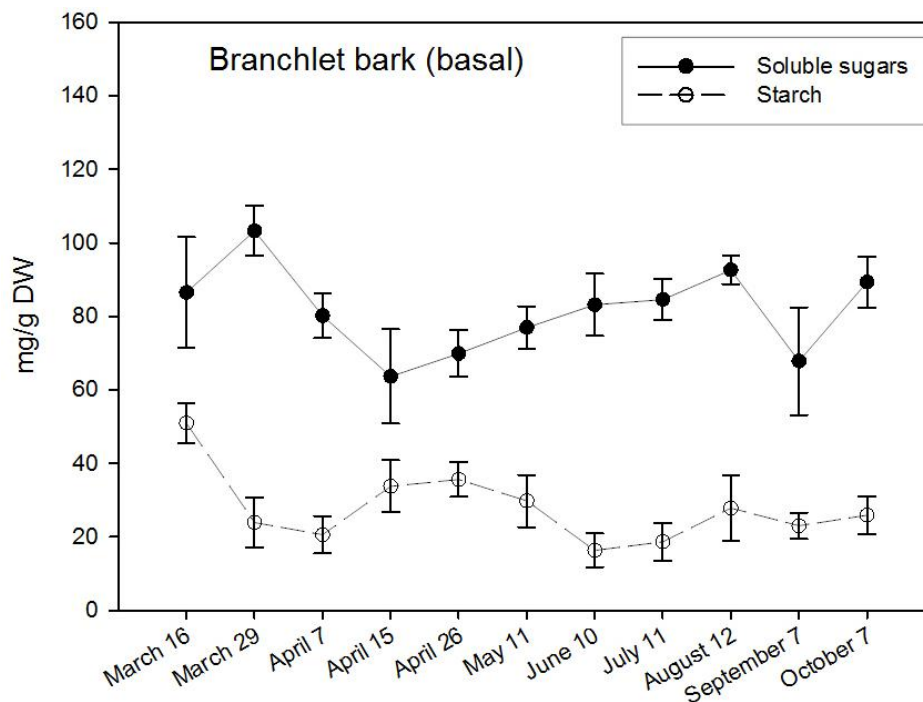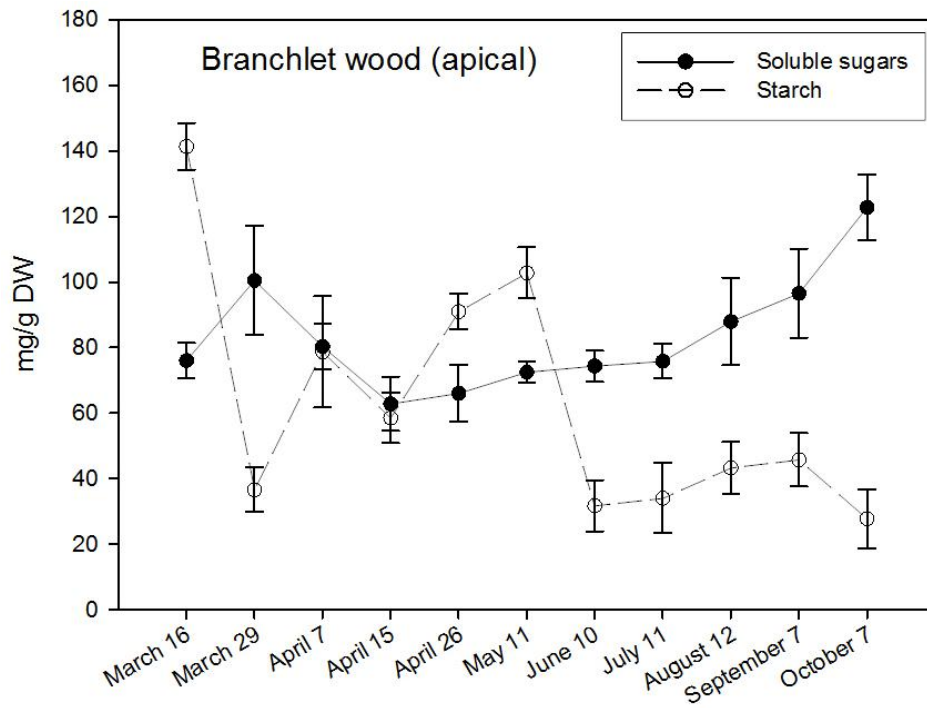

Fig. 3.5 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in bark of the basal part of the branchlet throughout the growing season from dormancy to harvest time (March – October).
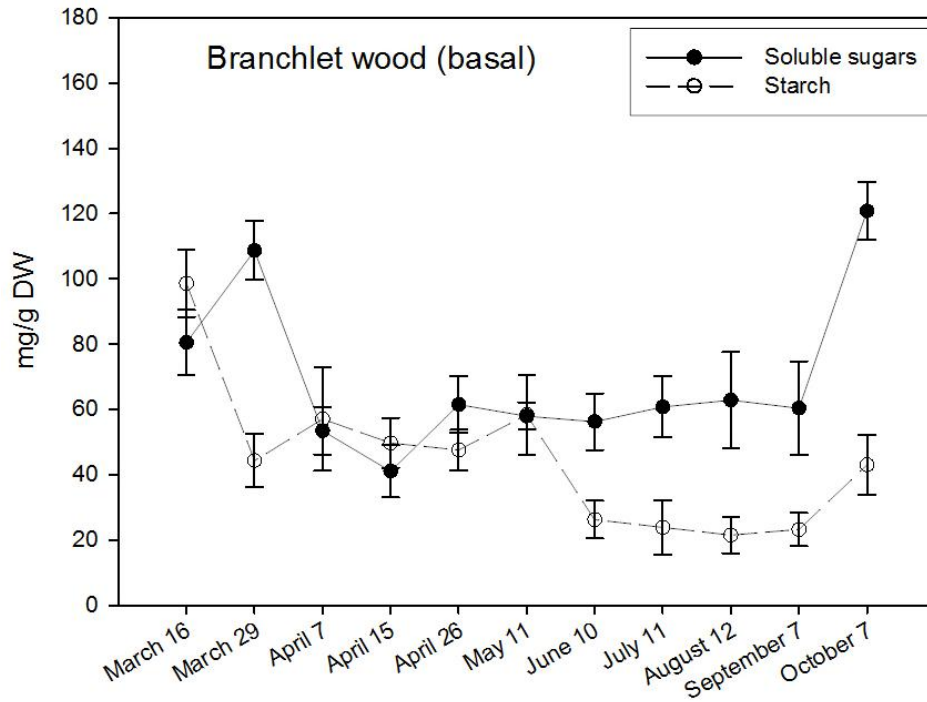
Fig. 3.6 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in woody part of the basal section of the branchlet throughout the growing season from dormancy to harvest time (March – October).

Fig. 3.7 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in bark of the apical part of the branchlet throughout the growing season from dormancy to harvest time (March – October).

Fig. 3.8 - Non-structural carbohydrates (NSCs – soluble sugars and starch) concentration (mg/g of dry weight) in woody part of the apical section of the branchlet throughout the growing season from dormancy to harvest time (March – October).

4. Conclusion

This preliminary study showed that seasonal carbohydrates balance (soluble sugars plus starch) in mature walnut trees is strongly correlated to the phenological stages, highly dynamic throughout the growing season and moreover among tissue organs.

We observed that in all tree compartments starch storage shifted during dormancy stage and its depletion occurred quickly before bud break, according to what was previously described in walnut and also in many deciduous tree species (Rinne *et al.*, 1994; Witt and Sauter, 1994; Bohhomme *et al*., 2005; Simões *et al.*, 2014; Charrier *et al*., 2015; Hartmann and Trumbore, 2016).

Such high mobilization of starch highlighted the essential energy requested to interrupt the bud dormancy and to support growth and metabolism of new organs at the beginning of the growing season.

The results shown here demonstrate that during intense growth and reproductive development fruits became the dominant sink; the strong reduction of stored energy observed in roots during the last phase of internal kernel highlighted fruits strength.

The consecutive starch deposition was weak and observed only in woody tissue of branchlets at the end of summer; probably this deposition continued later, before the leaf abscission in autumn.

In conclusion, the preliminaries data gained in this study can be used to improve the knowledge concerning the allocation and storage processes inside walnut trees and consequently the carbon source–sink relationships. Future additional data should contribute to complete the understanding of carbohydrates balance in walnut responding to environmental condition that may be of key importance to yield predictions, determination of plant reserve level and phenology.

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General conclusion

This study revealed additional knowledges related to the positive effect of irrigation on physiological and productive aspects of pistachio trees; moreover the obtained data on carbohydrates in walnut trees increased the information about photoassimilates mobilization responding to phenological stages.

Ψ_{SWP} was confirmed an efficient parameter to monitor plant water status and a useful tool to schedule irrigation. The water relations and photosynthetic parameters resulted dynamic, changing throughout the growing season. These results suggest the necessity to manage irrigation taking into consideration both water status and phenological stages of nut growth and development. From the relationship found between water status, physiological and photosynthetic parameters was confirmed a Ψ_{SWP} value, around -1.5 MPa, that could represent the transition point between mild and severe water stress condition and so it can be used as indicator for irrigation scheduling.

This study further showed that irrigation can positive influence the concentration of the chlorophylls a and b, that represents an important eye appeal traits and a discriminating element to differentiate cultivars and area of growing. Moreover, irrigation improved the sensorial attributes that cause turpentine, resin, green and balsamic aroma of pistachio kernel (terpenes class).

Concerning the walnut's carbohydrates mobilization and storage, emerged the strongly dynamism of photo-assimilates in relation to different plant compartments and phenological stages; further our preliminaries data confirmed the well-known carbon source–sink relationships showing a starch depletion in all tree compartments to interrupt the bud-dormancy and again during reproductive development when fruits became the dominant sink. The high mobilization of starch highlighted the essential energy requested to interrupt the bud-dormancy and to support growth and metabolism of new organs at the beginning of the growing season

Overall future researches are surely necessary to increase knowledge concerning the positive effect of irrigation on physiological and productive aspects in pistachio tree; aims to manage irrigation schedule improving the water efficiency, especially in an environment characterized by low water availability particularly for agricultural purposes. To increase knowledge about the alternate bearing phenomena in pistachio trees it is necessary to continue studying the competitive relationships between organs for the available resources. Finally additional studies should further contribute to complete the understanding concerning the allocation and storage processes in walnut responding to environmental condition that may be of key importance to yield predictions, determination of plant stress level and phenology.

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