Osmotic drought stress influence on physiological and biochemical characteristics of pistachio (*Pistacia* spp.) seedlings

Ali Esmaeilpour

.

In The Name Of Allah, The Most Beneficent, The Most Merciful

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Cover illustration:

***Front page*:**

Adult pistachio tree

Back page:

Top left: male flowers

Middle left: female flowers

Top right: ripen fruits

Bottom left: fruits in shell

Bottom right: pistachio kernels

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Regards,
Ali Esmaeilpour
Ghent, Belgium
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Summary

Pistachio belongs to the Anacardiaceae family. Only *Pistacia vera* L., i.e. cultivated pistachio, has economic importance. Iran, as the region of origin of pistachio, has always had the largest cultivation area in the world. About 80 percent of Iran is categorized as semi-arid and arid. In Iran, pistachios are usually cultivated under dry and saline conditions as they have a high drought and salinity tolerance. Still, water deficit and salinity can cause a reduction in growth, yield and nut quality. Drought stress adversely affects growth, dry mass accumulation, and productivity of plants, and causes a higher rate of impairment than any other environmental factor.

There is wide variation in edible pistachio (*P. vera*) cultivars in Iran which are grown under different environmental conditions. Yet, physiological responses of some pistachio cultivars and rootstocks to drought stress have hardly been studied. The aim of this study was to evaluate the effects of osmotic drought stress and subsequent recovery on physiological performance of three pistachio rootstocks (*P. vera* L. cv. Badami, *P. vera* L. cv. Sarakhs and *P. terebinthus*) and three of the country's most common *P.vera* cultivars (Akbari, Kaleghochi and Ohadi). In this respect, we conducted two experiments during a two years period in the glasshouse in Ghent, Belgium.

In the first year, three pistachio rootstocks, i.e., *P. vera* L. cv. Badami, *P. vera* L. cv. Sarakhs and *P. terebinthus* were subjected to four osmotic drought stress treatments: -0.1 (control), -0.5, -1.0 and -1.5 MPa using PEG 6000. Results obtained from this experiment (Chapter 3) indicate that all drought stress treatments decreased maximum quantum yield of PSII (F_v/F_m), effective PSII quantum yield (YII) and photochemical quenching (qP).

A decreasing osmotic potential of the nutrient solution significantly decreased leaf phosphorous (P) concentration compared to control plants. Effects of rootstocks on leaf mineral element contents varied significantly with drought stress condition.

Drought stress significantly decreased both plant fresh and dry weight, shoot and root dry weight, leaf area and stem elongation. There was also a significant rootstock effect on these growth parameters under drought stress condition. Under osmotic stress treatments, root/shoot ratio increased significantly. Control plants showed normal elongation growth, but growth was stopped with all drought stress treatments, and differences were significant for all three rootstocks. After two weeks of stress, a recovery of 2 weeks was applied. This period was insufficient to fully restore the negative effects of the applied severe stress on the studied rootstocks.

The applied osmotic drought stress on pistachio rootstocks induced significant reductions in chlorophyll fluorescence parameters, reduction in leaf nutrient content and increases in the leaf carbon isotope composition in pistachio rootstocks. Our results in this experiment show that *P. terebinthus* rootstock had better tolerated the applied drought stress as shown by the higher growth parameters performance in drought stress condition and lower carbon isotope composition as compared with *P. vera* L. Badami and Sarakhs rootstocks. YII could be used to distinguish the different response to drought stress in the studied pistachio rootstocks. As YII was affected after drought stress period, this parameter has a potential to be the early and non-destructive tool to screen pistachio rootstocks for drought tolerance.

In the second experiment, the three pistachio cultivars, i.e., Akbari, Kaleghochi and Ohadi, were subjected to three osmotic drought stress levels: control (-0.1 MPa), moderate (-0.75 MPa) and severe drought (-1.5 MPa) stress using PEG 6000 for a 14 day period. Our results (Chapter 4) showed that all drought stress treatments decreased net photosynthesis (*Pn*), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) of pistachio cultivars tested, but also that Ohadi cultivar better maintained its photosynthetic capacity compared to Akbari and Kaleghochi under drought stress conditions. Maximum quantum yield of PSII (F_v/F_m), effective PSII quantum yield (YII) and photochemical quenching (qP) were also reduced. Chlorophyll fluorescence parameters indicated that Akbari was more susceptible to the applied drought stress. Drought stress levels decreased chlorophyll pigments levels, fresh and dry weight, stem elongation, leaf nitrogen content (N), and leaf water potential, and increased water use efficiency (WUE). Proline strongly increased under drought stress for Akbari. After two weeks of stress, a recovery of 2 weeks was applied. This period was insufficient to fully restore the negative effects of the applied stress on the studied cultivars. Based on reduction of photosynthesis and increase of proline content, Akbari seems more sensitive to the applied drought stress.

Among pistachio cultivars, Ohadi had higher amounts of plant dry weight (biomass) and root dry weight under drought conditions than Akbari (significant) and Kaleghochi (non-significant).

Results obtained from the present study (Chapter 5) also revealed that carbohydrate accumulation varied with drought stress levels and pistachio cultivars. Both drought stress treatments increased carbohydrate and starch values. Sucrose amounts significantly increased with drought stress in all three cultivars, whereas glucose contents did not change

significantly. Relative water content (RWC) varied between pistachio cultivars. However, during the drought period, there were significant differences in the relative water content between drought stress levels and their respective control for Kaleghochi.

Drought stress significantly increased water use efficiency of treated seedlings for all cultivars. Stomatal numbers were significantly affected by the highest drought stress level in Kaleghochi; which was in contrast with non-significant changes in Akbari and Ohadi.

The impact of drought stress treatments on carbon isotope composition ($\delta^{13}C$) was not significant compared to the control in pistachio cultivars. There were no significant correlation between $\delta^{13}C$ and intrinsic and instantaneous WUE, biomass, *Pn* and soluble sugars in this experiment. Nitrogen isotope composition ($\delta^{15}N$) decreased in both drought stress treatments (-0.75 and -1.5 MPa) compared to the control.

Our study showed that different pistachio rootstocks and cultivars apply different mechanisms to deal with drought stress: drought avoidance and drought-tolerance mechanisms. Taking all ecophysiological and biochemical parameters obtained in this evaluation into consideration, it can be concluded that Terebinthus (*P. terebinthus*) rootstock and Ohadi (*P. vera*) cultivar are more tolerant to lower water availability in comparison with the other evaluated pistachio genotypes.

SAMENVATTING

Pistache behoort tot de familie Anacardiaceae. Alleen *P. vera* L., d.w.z. gecultiveerde pistache, heeft een significant economisch belang. Iran, de oorsprongsregio van pistache, is ook d het grootste teeltgebied ter wereld. Ongeveer 80 procent van het landoppervlakte in Iran is gecategoriseerd als halfdroog of droog. In Iran worden pistachenoten gewoonlijk onder droge omgevings- en mineraalrijke bodemomstandigheden geteeld, omdat ze een hoge tolerantie hebben tegen droogte, en zoute bodems en/of grondwater. Toch kan watertekort en saliniteit leiden tot een vermindering van de groei, de opbrengst en de nootkwaliteit. Droogtestress heeft een nadelige invloed op de groei, de accumulatie van droge massa en de productiviteit van de planten en veroorzaakt een sterkere productievermindering dan elke andere omgevingsfactor.

Er is grote variatie in eetbare pistachio (*P. vera*) cultivars in Iran die onder verschillende omgevingsomstandigheden worden gekweekt. Toch zijn de fysiologische reacties van sommige pistache cultivars en onderstammen op droogtestress nauwelijks bestudeerd. Het doel van deze studie was om de effecten van osmotische droogtestress en het daaropvolgende herstel te evalueren op de fysiologische prestaties van drie pistache-onderstammen (*Pistacia vera* L. cv. Badami, *P. vera* L. cv. Sarakhs en *P. terebinthus*) en drie van de in Iran, meest voorkomende *P.vera* cultivars (Akbari, Kaleghochi en Ohadi). In dit verband hebben we gedurende twee jaar twee experimenten uitgevoerd in de serres van de Faculteit Bio-ingenieurswetenschappen van de Universiteit Gent (België).

In het eerste jaar, werden drie pistachio onderstammen, d.w.z. *Pistacia vera* L. cv. Badami, *P. vera* L. cv. Sarakhs en *P. terebinthus* onderworpen aan vier osmotische droogtestressbehandelingen: -0.1 (controle), -0.5, -1.0 en -1.5 MPa met behulp van PEG 6000. De resultaten verkregen uit dit experiment (Hoofdstuk 3) geven aan dat bij alle droogtebehandelingen de kwantumopbrengst van PSII fotochemie (Fv/Fm), effectieve PSII kwantumopbrengst (YII) en photochemicsche quenching (qP) verminderden.

Een afnemend osmotisch potentieel van de voedingsoplossing zorgde voor de significante vermindering van de het fosforgehalte in bladeren in vergelijking met controle planten. Effecten van onderstammen op de minerale gehalte van de bladeren varieerden aanzienlijk volgens droogtestresscondities.

Droogtestress verminderde zowel het totale vers- als drooggewicht, het zdrooggewicht van stengels en wortelen, de bladoppervlakte en de stengelverlenging. Er was ook een significant onderstameffect op deze groeiparameters onder de droogtestresscondities. Onder

osmotische stressbehandelingen steeg de wortel/stengelverhouding aanzienlijk. Controleplanten vertoonden normale verlengingsgroei, maar de groei werd gestopt bij alle droogtebehandelingen, en de verschillen waren significant voor de drie onderstammen. Na twee weken stress werd een herstelperiode van 2 weken toegepast. Deze periode was onvoldoende om de negatieve effecten van de toegepaste ernstige stress op de onderzochte onderstammen volledig te herstellen.

De toegepaste osmotische droogtestress op de pistache onderstammen veroorzaakte significante verlagingen van de chlorofylfluorescentieparameters, een vermindering van het bladnutriëntengehalte en verhogingen in de bladkoolstof-isotoopsamenstelling. De resultaten van dit experiment laten zien dat de *Pistacia terebinthus* onderstammen de toegepaste droogtestress beter tolereerden, zoals blijkt uit de hogere groeiparametersprestaties in de droogte-stressconditie en de lagere koolstofisotopenamenstelling, in vergelijking met *P. vera* L. Badami en Sarakhs onderstammen. YII kan worden gebruikt om de verschillende reacties op droogtestress in de onderzochte pistache onderstammen te onderscheiden. Aangezien YII na de droogtestressperiode was aangetast, kan deze parameter een vroeg en niet-destructief hulpmiddel zijn om pistache onderstammen te screenen voor droogtetolerantie.

In het tweede jaar werden de drie pistachecultivars, d.w.z. Akbari, Kaleghochi en Ohadi, onderworpen aan drie osmotische droogtestresscondities: controle (-0.1 MPa), matige (-0.75 MPa) en ernstige droogtestress (-1.5 MPa) a.d.h.v. PEG 6000 gedurende een periode van 14 dagen. Onze resultaten (hoofdstuk 4) laten zien dat alle droogtestressbehandelingen de netto fotosynthese (Pn), de stomatale geleidbaarheid (gs), de intercellulaire CO_2-concentratie (Ci) en de transpiratiesnelheid (E) van de pistachekultivars verminderen, maar ook dat de Ohadi-cultivar een hogere fotosynthetische capaciteit had ten opzichte van Akbari en Kaleghochi, onder droogtestressomstandigheden. Maximale kwantumopbrengst van PSII fotochemie (Fv/Fm), effectieve PSII kwantumopbrengst (YII) en fotochemische quenching (qP) werden ook verminderd. Chlorofylfluorescentie parameters hebben aangetoond dat Akbari gevoeliger is voor de toegepaste droogtestress. De droogtesterkte verlaagde het gehalte aan chlorofylpigmenten, vers en droog gewicht, stengelverlenging, bladstikstofgehalte (N) en het bladwaterpotentieel en verhoogde de watergebruiksefficiëntie (WUE). Proline groeide sterk onder droogtestress voor Akbari. Na twee weken stress werd een herstelperiode van 2 weken toegepast. Deze periode was onvoldoende om de negatieve effecten van de toegepaste stress op de bestudeerde cultivars volledig te herstellen. Op basis van de verlaging van de

fotosynthese en deverhoging van het prolinegehalte lijkt Akbari gevoeliger voor de toegepaste droogtestress.

Onder de pistachecultivars had Ohadi een hoger totaal drooggewicht (biomassa) en worteldrooggewicht onder droogteomstandigheden dan zowel de Akbari (significante verschillen) als de Kaleghochi (niet-significante verschillen) cultivars.

Uit onze resultaten (Hoofdstuk 5) bleek ook dat accumulatie van koolhydraten sterk varieert tussen droogtestressniveaus en pistachecultivars. Beide droogtebehandelingen verhoogden de koolhydraat- en zetmeelgehaltes. Sucrosehoeveelheden werden significant verhoogd onder droogtestressomstandigheden in alle drie de cultivars, terwijl de glucose-inhoud niet significant veranderde. Het relatiefve watergehalten variëren tussen de pistache cultivars. Tijdens de droogteperiode waren er echter significante verschillen in het relatieve watergehalte tussen de planten onderworpen aan droogtestress en hun respectievelijke controleplanten bij de Kaleghochi cultivar.

Door droogtestress is de watergebruiksefficiëntie van de behandelde zaailingen voor alle cultivars aanzienlijk verhoogd. De aantallen stomata werden significant beïnvloed door het hoogste droogte-stressniveau in Kaleghochi; dit is echter in tegenspraak met de niet-significante veranderingen in Akbari en Ohadi cultivars.

Ons onderzoek toont aan dat verschillende pistache onderstammen en cultivars op verschillende manieren met droogtestress omgaan: droogte vermijding en droogtetolerantie mechanismen. Wanneer we alle ecofysiologische en biochemische parameters uit onze studie in rekening brengen, kunnen we besluiten dat de Terebinthus (*P. terebinthus*) onderstam en de Ohadi (*P. vera*) cultivar meer tolerant zijn voor lage waterbeschikbaarheid in vergelijking met de andere bestudeerde pistache genotypes.

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List of abbreviations

ATP- adenosine triphosphate

ABA- abscisic acid

ANOVA- analysis of variance

AK- Akbari

C_a- ambient CO_2

Ca- calcium

Car- carotenoid

CAT– catalase

Chl fluorescence- chlorophyll fluorescence

Chl a- chlorophyll a

Chl a+b- total chlorophyll

Chl b- chlorophyll b

C_i- intercellular CO_2

C isotope- carbon isotope composition

D- diameter

Dr. - drought stage

E- transpiration rate

ETR- electron transport rate

Fe- iron

F_m- maximum fluorescence yield

F_v- variable fluorescence

F_v/F_m - maximum quantum yield of PSII

F'_m - maximum fluorescence in ambient light

Ft- steady fluorescence in ambient light

GB- glycine betaine

GPX- glutathione peroxidase

GR- glutathione reductase

g_s- stomatal conductance

GSH- reduced glutathione

H- height

H_2O_2- hydrogen peroxide

HO•- hydroxyl radical

K- potassium

KA- Kaleghochi

LA- leaf area

LDW- leaf dry weight

LI- light intensity

Mg- magnesium

N- nitrogen

NPQ- non-photochemical quenching of chlorophyll fluorescence

O_2 - singlet oxygen

O_2•- - superoxide radical

OA- osmotic adjustment

OH- Ohadi

P- phosphorous

PAR- photosynthetic active radiation photosystem I

PAM- Pulse-Amplitude-Modulated

PDW - plant dry weight, biomass

PEG- polyethylene Glycol

PFW- plant fresh weight

Pn- net photosynthesis

PSII- photosystem II

QP- photochemical quenching of chlorophyll fluorescence

Re.- recovery stage

R/S- root /shoot ratio

RDW- root dry weight

RH- relative humidity

ROS- reactive oxygen species

RWC- relative water content

SA- salicylic acid

SDW- shoots dry weight

SE- standard error

SEL- stems elongation

SD- standard deviation

Si- silicon

SL- stomata length

SN- stomatal number

ST- starch

SW- stomata width

T- temperature

T_l- temperature of leaf thermocouple

VPD_l- vapour pressure deficit based on leaf temperature

WUE- water use efficiency

Zn - zinc

YII- effective quantum yield of PSII electron transport

Ψl- leaf osmotic potential

Ψs- osmotic potential of nutrition solution

Ψw- leaf water potential

$\Delta^{13}C$- carbone isotope discrimination

$\Delta^{15}N$- nitrogen isotope discrimination

$\delta^{13}C$- carbon isotope composition

$\delta^{15}N$- nitrogen isotope composition

List of Publications

A1/ ISI publications (published)

1. Esmaeilpour, Ali; Van Labeke, Marie-Christine; Samson, Roeland; Van Damme, Patrick. 2015. Osmotic stress affects physiological responses and growth characteristics of three pistachio cultivars. *Acta Physiologiae Plantarum* 37(6):1-14.

2. Esmaeilpour, Ali; Van Labeke, Marie-Christine; Samson, Roeland; P. Boeckx; Van Damme, Patrick. 2016. Variation in biochemical characteristics, water status, stomata features, leaf carbon isotope composition and its relationship to water use efficiency in pistachio (*Pistacia vera* L.) cultivars under drought stress condition. *Scientia Horticulturae* 211:158-166.

Papers in A2 journals (published):

1. Esmailpour, Ali; Van Labeke, Marie-Christine; Samson, Roeland; S. Ghafaripour; Van Damme, Patrick. 2015. Comparison of biomass production-based drought tolerance indices of pistachio (*Pistacia vera* L.) seedlings in drought stress. *International Journal of Agronomy and Agricultural Research*. Vol. 7, No. 2, p. 36-44.

Presentations or posters at international conferences

1. Esmaeilpour, A**.** and P. Van Damme. 2014. Evaluation of Seed Soaking Times on Germination Percentage, Germination Rate and Growth Characteristics of Pistachio Seedlings. The 29th international horticultural congress (IHC2014). Brisbane, Australia.

2. Esmaeilpour, A., M. C. Van Labeke, R. Samson and P. Van Damme. 2014. Variation of Relative Water Content, Water Use Efficiency and Stomatal Density during Drought Stress and Subsequent Recovery in Pistachio Cultivars (*Pistacia vera* L.). The 29th international horticultural congress (IHC2014). Brisbane, Australia.

3. Esmaeilpour, A.**,** M. C. Van Labeke, R. Samson and P. Van Damme. 2014. Chlorophyll fluorescence as a tool for evaluation of drought stress tolerance in Iranian

pistachio cultivars. International conference on research on food security, natural resource management and rural development (Tropendag 2014). September 17-19, 2014. Prague, Czech Republic.

4. Esmaeilpour, A.**,** M.C. Van Labeke, P. Boeckx and P. Van Damme. 2015. Impact of osmotic drought stress on carbon isotope discrimination and growth parameters in three pistachio rootstocks. XVI GREMPA Meeting on Almonds and Pistachios. May 12-14, 2015. Meknes, Morocco.

Chapter 1. **A review of global pistachio production**

1.1 Introduction

Iran, officially the Islamic Republic of Iran, is a country in West Asia. It is located between latitudes 25º 40' N and 39º 45' and longitudes 44º 15' and 62º 40' E. Iran shares its northwestern borders with Armenia and Azerbaijan. Across the Caspian Sea lie its other neighboring countries of Kazakhstan and Russia. To the northeast, it is bordered by Turkmenistan; to the east by Afghanistan and Pakistan; to the south by the Persian Gulf and the Gulf of Oman; and finally to the west by Turkey and Iraq. Comprising a total land area of 1,648,195 km^2, it is the second-largest nation in the 'Middle East' and the 18th-largest country in the world. Its total population is estimated to be around 78.3 million; consisting of various tribes, including Pars (the majority), Turk, Lur, Kurd, Baluch and Arab (the minority). Iran ranks as 17^{th} on the list of most-populous nations (Statistical center of Iran, 2014). Iran has vast amounts of fuel resources, such as petroleum, natural gas and coal. Other mineral resources include chromium, copper, iron ore, lead, manganese, sulfur and zinc. Iran is prone to and has in the past suffered from some (minor) natural disasters, which include periodic droughts, floods, dust storms, sandstorms and earthquakes.

Iran's climate ranges from arid or semi-arid in most of the country to subtropical along the Caspian coast and the northern forests. On the northern edge of the country (the Caspian coastal plain), temperatures rarely fall below freezing point and the area remains humid for the largest part of the year, while temperatures in summer rarely exceed 29 °C.

In Iran, rainfall is seasonal and mainly concentrated in winter; starting from late autumn and continuing throughout winter to end in early spring. For summer crops, irrigation is required in the whole country (Fardooei, 2001). Mean annual precipitation is 680 mm in the northern (humid) areas of the country, while the arid and semi-arid regions of the country (Figure 1.1) receive an average precipitation of less than 240 mm per year. Toward the east and center of Iran, precipitation decreases to 100 mm or less per year (Kousari and Ahani, 2012). Desert regions have evaporation that exceeds precipitation during most of the year. The vegetative cover is low and plant stands are sparse. Xerophytic plants such as some species of cacti, euphorbias, pine and etc. are the dominant plants in the Iranian deserts (Fardooei, 2001).

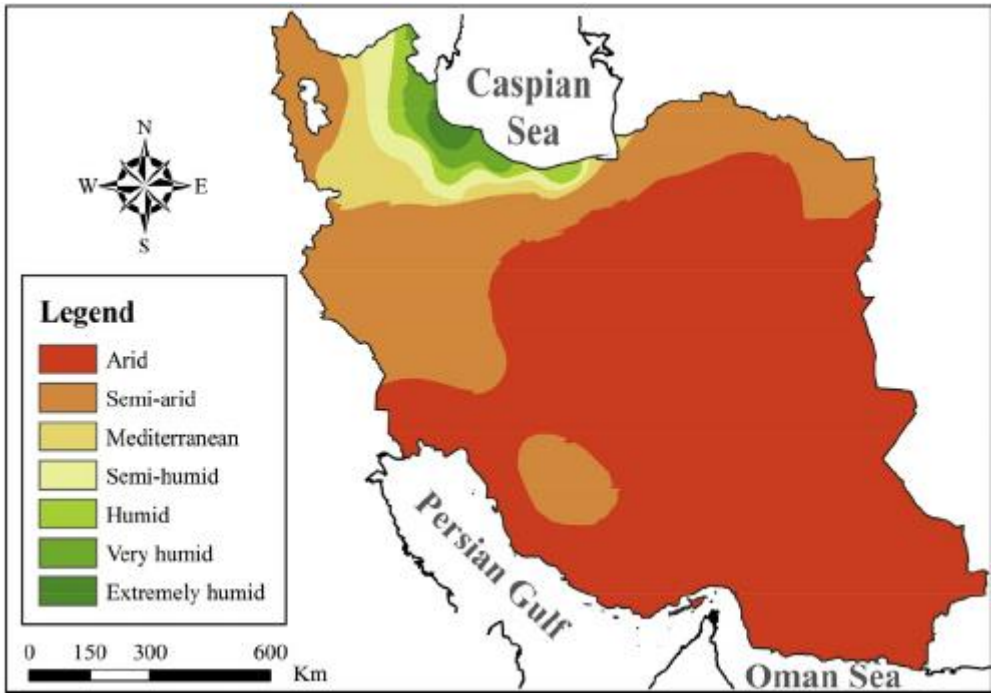


Figure 1.1: Climatology map of Iran (Peel *et al.*, 2013).

As mentioned before, there are various climates in Iran and each climate is suitable for specific crops. Arid and semi-arid climates cover approximately 80 percent (Figure 1.1) of the country's areas (Cheraghi, 2004; Fardooei, 2001; Kousari and Ahani, 2012). Water deficiency and salinity are the two most common factors that limit quality and quantity of crop production in latter areas (Abbaspour *et al.*, 2012a; Banakar and Ranjbar, 2010; Fardooei, 2001; Hajiboland *et al.*, 2014; Rouhi, 2007). These two factors also have a determining role in the distribution and diversity of crop plants (Abrishami, 1995). Therefore, agricultural activities are concentrated around the crops that are compatible with these specific regional conditions.

Wheat (*Triticum aestivum*), barley (*Hordeum vulgare)*, alfalfa (*Medicago sativa*), cotton (*Gossypium hirsutum*), sugar beet (*Beta vulgaris*), saffron (*Crocus sativus*), turnip (*Brassica rapa*), sunflower (*Helianthus annuus*), melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) are the most important crops produced in arid and semi-arid regions. Fruit crops in these regions are pistachio (*Pistacia* spp.), date (*Phoenix dactylifera*), citrus (*citrus* spp.), fig (*Ficus carica*), pomegranate (*Punica granatum*), almond (*Prunus dulcis*), apple (*Malus domestica*), olive (*Olea europaea*) and mulberry (*Morus alba*). The most important crop in these areas is pistachio (Figure 1.2), which plays an important role in the country's economy,

as it is called ‘the green gold of the desert’ (Abrishami, 1995; Esmaeilpour *et al.*, 2010; Panahi *et al.*, 2002).

Figure 1.2: Pistachio (*Pistacia vera*) production areas in Iran (Source: Iran Pistachio Aria Co. Ltd).

1.2 Importance of pistachio

The name pistachio comes from a very old Iranian term (usually pronounced as Pesteh in Farsi). The origin of the word is associated with the first area pistachio trees were domesticated. The term has been derived from the dialect of the people who lived in Khorasan in northeast of Iran in that time (Abrishami, 1995). The name of the pistachio in most world languages is therefore derived from the Persian word (Pesteh) (Abrishami, 1995; Sheibani, 1995).

The most important pistachio producing countries in the world are: Iran, the US, Turkey, China and Syria (Figure 1.3). Iran is the world’s largest pistachio producer and exporter. It is an important horticultural crop in Iran, as pistachio orchards (bearing and non-bearing trees) cover more than 450,000 ha of the country, yielding an annual production of about 255,000 tons, from some 60 different varieties (Anonymous, 2014; Banakar and Ranjbar, 2010; Bastam *et al.*, 2013; Esmaeilpour and Khezri, 2006; Nooghi and Mozafari, 2012; Sheibani, 1995). Although the areas planted for pistachio increased significantly during 2000–2012

(See appendix 1- chapter 1), the average dry yield (kg/ha) of pistachio orchards decreased during the same mentioned period (See appendix 2- chapter 1). Inadequate irrigation (drought stress) and high salinity level of agricultural water are the main restrictions that have affected the pistachio productivity in current years in Iran.

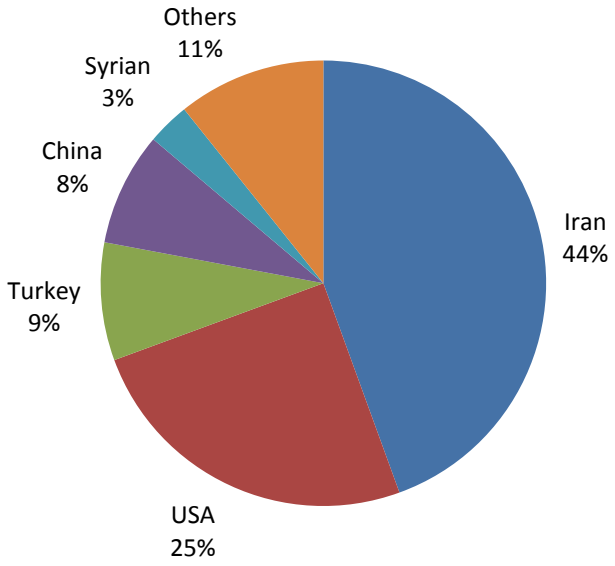


Figure 1.3: Major pistachio producing countries in the world based on their relative contribution to the world pistachio nut production (FAO 2012).

Pistachio is cultivated in Iran's regions with low rainfall of nearly 100 mm per year, with a wide temperature range between -20 °C in winter to 42 °C in summer. Pistachio plantations occupy large areas in many parts of Iran, i.e. southeast (Kerman, Yazd, Fars, Sistan and Baluchistan provinces), central (Esfahan, Qom, Tehran and Qazvin provinces), northeast (Semnan, Golestan and Khorasan Razavi provinces) and northwest (East and west Azerbaijan provinces). Irrigation deficit and high salinity level of irrigation water are the main limitations experienced by pistachio farmers in arid and semi-arid areas (Hajiboland et al., 2014; Sedaghat, 2010).

As arid and semi-arid regions are rendered unsuitable for the economically beneficial production of other crops, pistachio remains the only economic feasible option for local farmers. In spite of the fact that in the current years, the productivity of pistachio orchards has been affected by the restriction parameters mentioned above, Iran still has the largest production areas, production and export rates among all other pistachio producing countries (Esmaeilpour et al., 2010; Panahi et al., 2002; Razavi, 2010; Sedaghat, 2010). Pistachio

production has the second position in Iran's economy in terms of exported values after petroleum, which is not a renewable resource. Economy experts in Iran want to increase the economic stability by increasing non-oil exports to other countries. In this respect, there are a number of alternatives to oil and one of the strongest is the improvement of pistachio's role in the country's economy (Abrishami, 1995; Esmaeilpour et al., 2010; Sedaghat, 2010).

Pistachio as the most important perennial fruit crop has an important role (Figure 1.4) in the economy of Iran (Abrishami, 1995; Anonymous, 2014; Razavi, 2010; Sedaghat, 2010). Pistachio nuts also have a high nutritional value compared to other nuts. They contain low concentrations of saturated fat and have a good source of mono-unsaturated fatty acids. Daily consumption of about 57 g of pistachio can decrease blood pressure in the elderly (Shakerardekani and Karim, 2013). Pistachio powder (obtained from the kernel) is used in confectionaries and its green skin pistachio fruit is used for feeding cows, sheep and poultry. Pistachio oil is used in producing cosmetics, and a large variety of culinary dishes and medicines (Razavi, 2010; Shakerardekani and Karim, 2013).

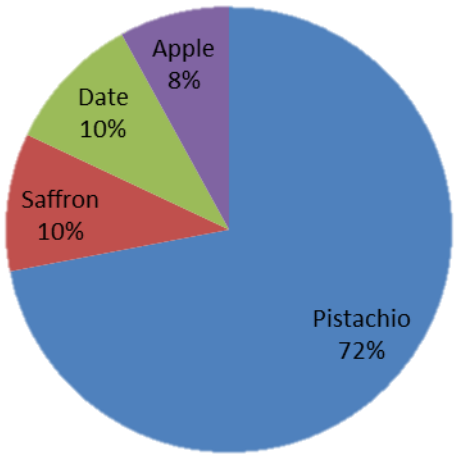


Figure 1.4: Shares of Iran's exported main crops in 2014 (Anonymous, 2014).

Overall, pistachios are appreciated for several reasons: (1) they have an economic importance, as they are exported both raw and roasted for their high nutritional values; (2) they are utilized in pharmaceuticals and the chemical industry; (3) the kernel, kernel slice and

powder are used in the confectionary industry, delicacies such as '*halva*', butter and milk are produced from its kernel; (4) they are a huge natural (forest) genetic resource for conservation; and finally (5) trees provide soil stabilization for soil erosion control.

1.3 Pistachio taxonomy

The genus *Pistacia* is a member of the Anacardiaceae, or cashew family. Other important members of this family include cashew (*Anacardium occidentale*), mango (*Mangifera* spp.), poison oak (*Toxicodendron vernicifluum*), and sumac (*Rhus coriaria*). *Pistacia* L. is mainly a subtropical genus and it consists of 11 species of dioecious trees and shrubs. All are characterized by pinnate leaves and single-seeded drupes (Zohary, 1996; Zohary, 1952). The term 'dioecious' refers to the fact that there are separate male and female trees and pollination is by wind (Esmaeilpour, 1998; Tajabadipour, 1997).

The most important species used as rootstock are *Pistacia vera* L., *P. atlantica* Desf., *P. integerrima* Rech.F, *P. terebinthus* L., *P. mutica* (Fisher), *P. khinjuk* Stock, *P. palestina* Boiss, *P. chinensis, P. lentiscus* L., *P. mexicana* HBK and *P. texana* Swingle (Zohary, 1952). The latter author performed the first complete taxonomic classification of the pistachio genus. However, today, Zohary's taxonomy is highly questionable regarding the status of many *Pistacia* spp., and the accuracy of dividing the genus into four sections. In the present study, we have used the classification of Al-Saghir and Porter (2012) which has divided the *Pistacia* genus in two sections (*Pistacia* and *Lentiscella*), nine species and five subspecies. *P. vera* L. is the only cultivated and commercially grown species in the genus (Zohary, 1996). Some of the other species are used as ornamentals and others as non-economic fruit trees. The first nine species are native to 'the old world'; two occur in southern USA and Mexico. Geographically, the largest concentration of *Pistacia* species is found in West Asia and the Mediterranean basin. Zohary (1996) performed the full-scale taxonomic treatment of *Pistacia*, using as the main diagnostic traits leaf characteristics and fruit morphology.

The genus *Pistacia* consists of xerophyte species (Belhadj *et al.*, 2011). Iran is one of its origin and diversity centers in the world (Arzani *et al.*, 2013; Esmaeilpour, 1998; Panahi et al., 2002; Pazouki *et al.*, 2010; Sheibani, 1995). In addition to the cultivated species (*P. vera* L.), Iran is also home to three wild species of pistachio i.e. *P. vera*, *P. khinjuk* and *P. atlantica*. The latter has three subspecies (*mutica, kurdica* and *cabolica*) (Khatamsaz, 1988). The characteristics of the pistachio species and cultivars that were used in our experiments are described in the following paragraphs.

1.3.1 *Pistacia vera* L.

The origin of *Pistacia vera* L. (pistachio) can be traced back to the Middle East and central Asia. It is one of the most important tree species grown in the Mediterranean climate areas of Iran, Syria, Lebanon, Turkey, Tunisia and Egypt (Zohary, 1996). The area of origin of domesticated *P. vera* is Khorasan Razavi province located in northeast Iran (Abrishami, 1995; Sheibani, 1995). All domestic pistachios (cultivars) and wild Sarakh (native) belong to this species (Esmaeilpour, 1998; Sheibani, 1995). Fruits of this species are large, edible and have great economic value. This species is generally grown as a tree (Panahi et al., 2002; Sheibani, 1995), with a height of between 3 to 5 m. The tree crown, especially in female plants spread. Leaves are compound and odd-pinnate. There are one or two pairs of leaflets and odd leaflets on the tip of the leaves, which are generally larger than the other leaflets. The leaves without stipules are dark green, the upper side is bright and the bottom dull-colored. The shape of the leaflets of the female trees is elliptic, whereas in male trees its shape is closer to ovate (Tajabadipour, 1997).

The inflorescence of *P. vera* is a panicle, staminate and pistillate flowers do not have petals. Staminate clusters have 200-300 flowers and pistillate clusters have 80-130 flowers (Esmaeilpour et al., 2010; Panahi et al., 2002). The fruits are drupes of 10-23 mm long and 6-12 mm wide (Tajabadipour, 1997). Male flowers of this species open earlier than female flowers (protandry), in early to mid-April in Iran (Esmaeilpour et al., 2010). The shape of the fruits varies from long elliptic to round (Tajabadipour, 1997). In Iran, the Badami variety is mostly used as rootstock, although in some areas, Ghazvini and Sarakhs are used as well (Esmaeilpour, 1998; Panahi, 2009). The chromosome number is 2*n* =30 in this species (Basr Ila *et al.*, 2003; Harandi and Ghaffari, 2001).

1.3.1.1 *P.vera* cv. Akbari

This variety is a high-vigor cultivar (Figure 1.5, A); nuts are long, elongated and large. The main characteristics of cultivar Akbari are: sparse clusters, high productivity, uniformity of fruit ripening, high vegetative growth, and large and numerous leaves (Esmaeilpour, 1998; Tajabadipour, 1997). Due to its high vegetative growth, this cultivar is suitable for regions with intense sunlight and hot summer temperatures in order to decline the effects of sunburn damages of pistachio fruits, stems and trunks (Esmaeilpour et al., 2010). Almost all its leaves are composed of three and five leaflets, whereas the cultivar also has single and 4-leaflet leaves (Tajabadipour, 1997). It is late-blooming; experiencing its blooming period in the

middle of April. As with other late-bloomers, leaves appear earlier than flowers in this cultivar (Esmaeilpour et al., 2010; Tajabadipour, 1997). This cultivar can be harvested at the end of September and is a late-ripening cultivar (Figure 1.5, B). The nuts are almond-shaped (Figure 1.5, C) and nut weight is 128.6-141.5 g per 100 nuts (Esmaeilpour et al., 2010; Tajabadipour, 1997). The average yield of this cultivar is 1205 kg of dried nut per ha in Rafsanjan area (Abdolahi, 2016).

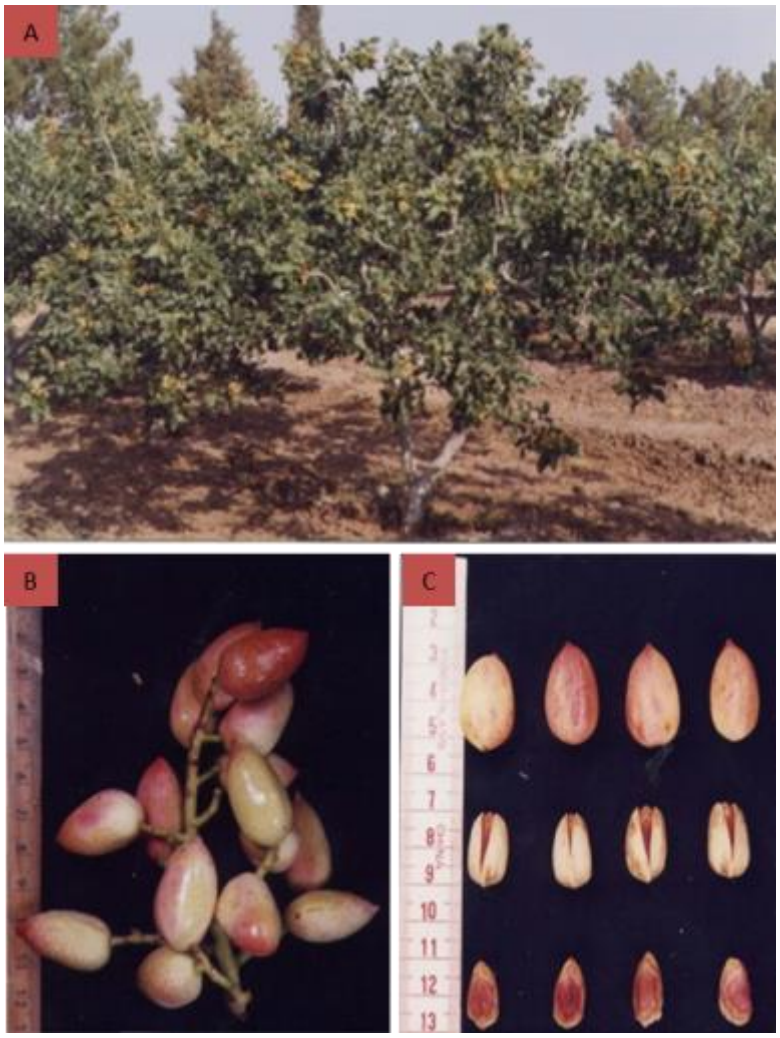


Figure 1.5: *P. vera* cv. Akbari tree (A), fruit cluster (B) and seeds (C) (Esmaeilpour, 1998).

1.3.1.2 *P.vera* cv. Badami- Zarand

This cultivar originates from the city of Zarand in Kerman province, where it was initially selected (Esmaeilpour, 1998; Sheibani, 1995). It has high growth vigor and a spreading

crown (Figure 1.6, A), reaching up to 3-5 m high. Almost all of its leaves are composed of three leaflets and the cultivar has no single or 4-leaflet leaves (Tajabadipour, 1997). Apical dominance is medium. Fruit cluster shape is medium (Figure 1.6, B) whereas ripening time occurs during early September. The nuts are almond-shaped (Figure 1.6, C) and nut weight is 79-83 g per 100 nuts (Esmaeilpour, 1998). This cultivar is used as a common rootstock (around 80 %) in pistachio orchards in Iran (Esmaeilpour et al., 2010).

Figure 1.6: *P. vera* cv. Badami-Zarand tree (A), fruit cluster (B) and seeds (C) (Esmaeilpour, 1998).

1.3.1.3 *P. vera* cv. Kaleghochi

This commercial cultivar of pistachio is widely used in both the Rafsanjan and Kerman areas. Its high yield and large fruits are the main reasons for its popularity (Figure 1.7, A). It has a more clear growth habit when compared with Ohadi, and supports stronger branches. It fruits the third year after grafting. The fact that it produces early blossom causes makes it susceptible to spring frost damage (EsmailPour, 1998; Sheibani, 1995). It also has higher branching compared with Ohadi (Esmaeilpour, 1996), medium growth vigor and a spreading crown. It grows up to 3.1 m in height and its apical dominance is weak. Most leaves are composed of five leaflets (Tajabadipour, 1997). Fruit cluster is medium shape (Figure 1.7, B) and ripening time occurs late when compared to Ohadi, in the beginning of September. Nuts (Figure 1.7, C) are round (hazelnut-shaped) and nut weight is 128.6-141.5 g per 100 nuts (EsmailPour, 1998; Tajabadipour, 1997).

Figure 1.7: *P. vera* cv. Kaleghochi tree (A), fruit cluster (B) and seeds (C) (Esmaeilpour, 1998).

1.3.1.4 *P. vera* cv. Ohadi

This is a very common cultivar, suitable for cultivation in most parts of Iran's pistachio production areas. It was first selected by a pistachio grower (Mehdi Ohadi) in Rafsanjan, in the 1941-1951 period (Sheibani, 1995). Its cultivation has consistently increased during the

past 40 years and now covers more than 50-60 % of cultivated pistachio orchards in the Rafsanjan and Kerman areas (Esmaeilpour et al., 2010; Sheibani, 1995). This cultivar has medium growth vigor and a spreading crown (Figure 1.8, A). It grows up to 3 m in height; its apical dominance is very high and most of its leaves are made up of 3-leaflets (Esmaeilpour, 1996; Tajabadipour, 1997). Fruit cluster is medium shape (Figure 1.8, B) and ripening time occurs around early September. Nut shape is round (Figure 1.8, C) and nut weight is 94.3 108.8 g per 100 nuts (Tajabadipour, 1997).

Figure 1.8: *P. vera* cv. Ohadi tree (A), fruit cluster (B) and seeds (C) (Esmaeilpour, 1998).

1.3.1.5 *P. vera* cv. Sarakhs

Sarakhs pistachio (*P. vera*) originated from Central Asia, near the border of Afghanistan, Turkmenistan and northeast Iran. This variety is widely distributed in the Khorasan (Khajeh Kalat, Shoricheh) and Golestan (Maraveh Tapeh) provinces (Abrishami, 1995; Sheibani, 1995), located at latitude 35°- 38°N and longitude 56°- 60°E, and at an altitude of 750- 1700 m above sea level, as wild (native) pistachio forest (Figure 1.9, A) (EsmailPour, 1998; Sheibani, 1995). The shape of the nuts is almond-like and small; an average nut weighs around 54.3- 62.8 g per 100 nuts (Figure 1.9, B). The total area planted with this variety is

about 20,000 ha (Sheibani, 1995). These trees are important in forest regions, because they provide protection from wind and water erosion, contribute to soil stability, provide fruit production and can be used as seed for cultivated pistachio rootstocks (Abrishami, 1995).

Figure 1.9: Natural forest of wild pistachio (*P.vera* var. Sarakhs) (A) and seeds (B) in Iran (Esmaeilpour, 1998).

1.3.2 *P. terebinthus* L.

This wild species is widely spread across the Mediterranean and more temperate areas (Ferguson *et al.*, 2005). The seedlings of *P. terebinthus* can grow in the stony and calcareous soil of the drier areas. They are resistant to cold and drought. For this reason, *P. terebinthus* seedlings, growing naturally in non-agricultural areas, could be grafted with pistachio cultivars and provide benefits of pistachio cultivation (Atli *et al.*, 1998). *P. terebinthus* grows as shrub, bush or small to medium tree. They may grow up to 3–5 m in height (Figure 1.10, A) depending on soil conditions (Atli et al., 1998; Ferguson et al., 2005; Zohary, 1996). Leaves of *P. terebinthus* are odd-pinnate; the number of leaflets varies, but usually has four to six pairs. The shape of the leaflets is ovate to lanceolate (Figure 1.10, B). The odd leaflet on the top of the leaf is similar in size to the other leaflets (Zohary, 1996). The number of chromosomes in this species is the same as for *P. vera* ($2n$ =30) (Basr Ila et al., 2003).

P. terebinthus shows variations with regard to flower color. In fact, the structure and color of the clusters may change from one plant to another. However, a cluster and its rachis usually have a reddish color. Flowering time of this species occurs at the same time of *P. vera*, and in many cases also with *P. atlantica* varieties. The shape of its fruits resembles a

swollen lentil (Figure 1.10, C), 5.7 mm long and 4.2 mm wide (Atli et al., 1998; Zohary, 1996).

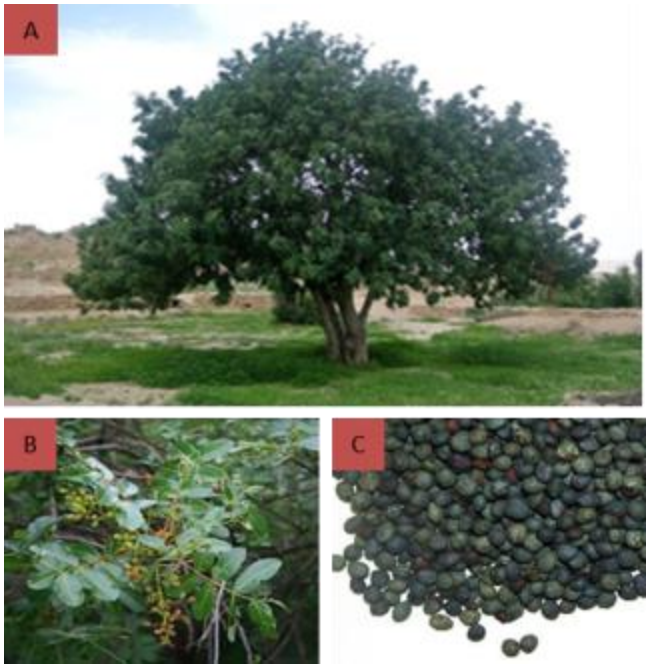


Figure 1.10: *P. terebinthus* tree (A), leaves and fruit cluster (B) and seeds (C).

1.4 Production problems

Water deficiency, salinity, and high lime concentration and thus soil pH, are the most common factors that limit quality and quantity of crop production in the arid and semi-arid regions of Iran. These factors also have a role in determining the distribution and diversity of crop plants over different areas (Fardooei, 2001; Kousari and Ahani, 2012).

Most pistachio plants, growing in arid and semi-arid regions, suffer from seasonal drought stress, saline irrigation water, seasonal frost (especially spring frost) stress, chilling and thermal requirements which are not met and heat stress during periods of active growth. Consequently, plants may be damaged or killed, resulting in lower nut yield and quality at harvest or even complete crop failure (Esmaeilpour et al., 2010; Pessarakli, 1994). Water deficiency is a common phenomenon during the pistachio crop cycle in most parts of Iran.

During seed germination, vegetative growth, flowering and kernel growth, pistachio is the most vulnerable to soil drought. Stress imposed during these stages drastically affects plant growth and crop yield (Panahi et al., 2002; Pessarakli, 1994).

Commercial pistachio cultivars which were used in this research i.e. Akbari, Kaleghochi and Ohadi have the ability to produce an annual average yield of 1205, 1075 and 1055 kg dry nuts per hectare, respectively (Abdolahi, 2016). The quality characteristics (nut shape and weight, clean shell, split percentage and blank percentage) of these 3 cultivars are better than that of the other Iranian cultivars, because these cultivars were selected according to these valuable characteristics by farmers over the last few decades.

In pistachio, water deficiency not only decreases tree growth and nut yield, but it also affects nut quality (decreased proportion of split nuts in comparison to nut size) and increases the alternate bearing intensity (Goldhamer and Beede, 2004; Kanber *et al.*, 1993). The level of injury or death of seedlings and adult pistachio trees as a result of severe and progressive drought stress conditions depends on the drought tolerance abilities of pistachio rootstocks and cultivars.

The potential impact of climate change will result in unreliable precipitation patterns and endanger the available quality and quantity of irrigation water (Razavi, 2012). Global climatic changes will have important effects on plant photosynthesis (Pessarakli, 1994). In addition, climate change and global warming will lead to warming up of the temperate zones, so that they also become suitable for subtropical crops like pistachio.

Pistachio plants can be used as an agricultural crop in arid and semi-arid areas, because they are tolerant to drought, soil and water salinity and high pH; in other words, conditions deemed inadequate for any other crop. On the other hand, as mentioned before there is a wide genetic variation among pistachio cultivars in Iran, so that they may have different responses ability to drought condition. Despite its potentially high drought tolerance and high genetic variation, responses of pistachio cultivars to drought stress have not been adequately determined.

1.5 Research objective

During the last fifty years, efforts have been done to develop pistachio cultivation areas in Kerman province (Iran) but global warming has caused increasing water deficiency and salinity in Kerman during the last decade, and consequently has decreased the pistachio production in this area. However, pistachio cultivation has been still extending in other suitable areas in Iran.

Akbari, Kaleghochi and Ohadi are commercial cultivars with good productivity (in terms of quality and quantity) and Badami-Zarand and Sarakhs are common rootstocks that are

used in Iran and *P.terebinthus* is one of pistachio species which is used as a rootstock for pistachio cultivars in Turkey, Greece, Italy and USA. There are limited studies about the suitability of *P.terebinthus* for development in Iran and other countries.

There is a general postulate that the pistachio tree is tolerant to drought. This claim is confirmed by observations done under natural conditions and by a few number of experimental studies on different rootstocks (Abbaspour *et al.*, 2011; Bagheri *et al.*, 2011; Habibi and Hajiboland, 2013; Panahi, 2009). Although adult pistachio trees are well-known for their drought tolerance, drought is one of the important environmental stresses that decreases the pistachio yield in Iran (Sedaghati and Hokmabadi, 2015). However limited evidence is available on the underlying mechanisms of drought tolerance associated with the plant's biochemical composition, physiological responses, water relation parameters, growth features and especially carbon and nitrogen isotope composition.

We hypothesize here that there is/are cultivar(s), among above mentioned pistachio genotypes, which is/are more tolerant to drought stress than others.

The main objectives of this study were to (1) identify the plant defence mechanisms against drought stress in pistachio rootstocks and cultivars (2) identify the most drought tolerant rootstock and cultivar under stress conditions (3) and identify suitable traits in order to screen rootstock and cultivar for their drought tolerance.

The specific objectives of these investigations were to:

(1) To determine chlorophyll fluorescence responses, leaf mineral contents, growth parameters and carbon isotope composition of three pistachio rootstocks i.e. *P. vera* cv. **Badami**, *P. vera* cv. **Sarakhs** and ***P. terebinthus*** during varying degrees of drought stress and subsequent recovery period, for the estimation of their growth potential as rootstock materials.

(2) To evaluate photosynthetic gas exchange patterns, biochemical responses, leaf water status, growth parameters, stomatal features, and carbon and nitrogen isotope composition of three important Iranian pistachio cultivars, i.e. *P. vera* cv. **Akbari**, *P. vera* cv. **Kaleghochi** and *P. vera* cv. **Ohadi**, under varying degrees of osmotic drought stress and subsequent recovery period, for the estimation of their growth potential as rootstock materials.

(3) And to identify a fast method to screen drought tolerant pistachio cultivars and rootstocks by using carbon isotope composition.

These investigations should ultimately lead to better-informed recommendations for the selection of drought tolerant cultivars and rootstocks.

Seedling rootstocks have improved pistachio tree growth and nut production (Esmaeilpour et al., 2010; Ferguson *et al.*, 2002a; Ferguson *et al.*, 2002b; Panahi et al., 2002). Therefore, researchers and growers have been focusing their efforts on selecting suitable rootstocks and cultivars for domestic pistachio trees. Selection of the best rootstock and cultivars, based on ecophysiological drought stress characteristics and growth parameters is of critical importance to optimize vegetation growth in dry environments.

Field studies of drought effects are difficult to perform, time-consuming and usually imprecise. An accurate control of soil water potential is also difficult to assess in the field. To overcome these problems, polyethylene glycol (PEG) has been used to maintain rooting media at predetermined water potential values.

1.6 Outline of thesis

Activities and results related to our objectives are spelled out here in 6 chapters. The first chapter covers the general review of pistachio both in Iran and the world. It focuses on important characteristics and the distribution of pistachio species and cultivars, and presents the research problems. Chapter 2 deals with the literature of abiotic drought stress and plant response to drought stress condition.

As indicated in first part of our specific objective, chapter 3 deals with the effects of osmotic drought stress on physiological responses (chlorophyll fluorescence parameters), growth characteristics (leaf, stem, root and total dry weight), mineral elements (P, Ca, Mg, Fe and Zn) and carbon isotope composition of three pistachio rootstocks i.e. *P. vera* cv. Badami, *P. vera* cv. Sarakhs and *P. terebinthus*, during varying degrees of drought stress and a subsequent recovery period. This chapter describes the mechanism involved in drought stress treatments in different pistachio species and how these rootstock seedlings overcome drought stress.

Chapter 4 focuses on osmotic stress and how it affects physiological responses and growth characteristics at different drought stress treatments in glasshouse for three pistachio cultivars. This chapter is related to our second objective and describes the effect of drought stress on photosynthetic gas exchange patterns, chlorophyll fluorescence parameters, pigments concentration and biomass content in pistachio cultivars i.e. *P. vera* cv. Akbari, *P. vera* cv. Kaleghochi and *P. vera* cv. Ohadi during drought stress and subsequent recovery weeks. The aim of this research was to evaluate the physiological mechanisms of drought tolerance of three pistachio cultivars. As we know not only the drought period in plants but

also water recovery by plants after the drought stress is very important. Therefore, this chapter investigates the mechanisms of drought stress on pistachio cultivars during drought stress and the subsequent recovery weeks.

In chapter 5 impact of osmotic drought stress on the biochemical characteristics, water relations, and nitrogen and carbon isotope composition of pistachio (*Pistacia vera* L.) cultivars are described. The aim of this research was to evaluate the mechanisms of drought stress tolerance according to biochemical responses on three pistachio cultivars with as the final goal of the development of suitable criteria for selecting drought tolerant cultivars. This chapter evaluates effects of drought stress on the accumulation of carbohydrate and starch, leaf water potential, and stomatal characteristics (first objective) as well as nitrogen and carbon isotopes composition (third objective) in the above-mentioned pistachio cultivars, during drought stress and subsequent recovery weeks. Finally, our general conclusions are discussed and recommendations are offered for future research in chapter 6.

Chapter 2. **Literature review**

2.1 Drought stress

Drought is a meteorological and environmental event, defined somewhat loosely as absence of rainfall for a long time period that causes depletion of soil moisture and damage to plants (Levitt, 1980; Turner, 1997). A similar definition has been brought up by Van Damme (1990), who stated that drought stress is a water deficit stress caused by a rain deficit that would make it a more meteorological term. When plant is exposed to an artificial water shortage, this is usually called desiccation (Levitt, 1980).

Drought is the most important environmental stress that severely impairs plant growth and development, limits plant production and the performance of crop plants more than any other environmental stress factor (Shao *et al.*, 2009). Plants experience drought stress either when the water supply to roots becomes impaired or when the transpiration rate becomes very high (Ali *et al.*, 2008; Jaleel *et al.*, 2009).

In agriculture, drought resistance is defined as the ability to grow, flower and produce economic yield with minimum yield loss under a water-deficit condition related to the well water management (Gupta, 1975; Kafi *et al.*, 2012; Levitt, 1980; Taiz and Zeiger, 2010).

2.2 Mechanism of drought resistance

In plants, the mechanisms of drought resistance can be categorized into three groups, i.e. drought escape, drought avoidance and drought tolerance (Mitra, 2001). However, crop plants use more than one mechanism at a time to resist drought (Belhassen, 1997; Blum, 1996; Kafi et al., 2012; Mitra, 2001; Rosielle and Hamblin, 1981).

2.2.1 Drought escape

Drought escape is defined as the ability of a plant to complete its life cycle or growing period before serious soil and plant water deficits develop (Mitra, 2001). This mechanism involves a rapid phenological development (early flowering and early maturity), developmental plasticity (variation in the duration of growth period depending on the extent of water-deficit) and remobilization of assimilates (Kafi et al., 2012; Mitra, 2001; Taiz and Zeiger, 2010). Duration to flowering is an important trait related to drought adaptation, whereby a short life cycle can lead to drought escape (Araus *et al.*, 2002).

2.2.2 Drought avoidance

Drought avoidance is the ability of plants to maintain relatively high tissue water potential despite a shortage of soil-moisture (Mitra, 2001). Drought avoidance is performed by the maintenance of turgor through increased rooting depth, efficient root system and increased hydraulic conductance and by reduction of water loss through reduced epidermal (stomatal and lenticular) conductance, reduced absorption of radiation by leaf rolling or folding (Levitt, 1980; Mitra, 2001) and reduced evaporation of the surface (leaf area) (Levitt, 1980; Turner, 1986a). Plants growing under drought conditions survive by doing a balancing act between maintenance of turgor and reduction of water loss (Levitt, 1980; Mitra, 2001).

Root morphological characteristics such as total biomass, length, density and depth are the main drought avoidance traits that contribute to final yield in severe drought stress (Subbarao *et al.*, 1995; Turner *et al.*, 2001). A deep and thickened taproot is helpful for extracting water from considerable depths (Kavar *et al.*, 2008). The response of isohydric plants to water shortage is a form of drought avoidance, as there is little initial relationship between soil water potential and leaf water potential (Limpus, 2009) .

2.2.3 Drought tolerance

Drought tolerance is the ability to withstand water-deficit conditions with low tissue water potential. The response of plants to tissue water-deficit determines their level of drought tolerance (Beck *et al.*, 2007; Farooq *et al.*, 2009b; Kafi et al., 2012; Levitt, 1980; Mitra, 2001). The mechanisms of drought tolerance are turgor maintenance through osmotic adjustment, maintenance of membrane integrity and metabolite accumulation (Chaves and Oliveira, 2004; Van Damme, 1990). As leaf water potential of anisohydric plants decreases under water shortage conditions, these plants apply tolerance mechanisms to cope with drought conditions (Limpus, 2009).

Hydraulic failure phenomena happen in plants, when reduced water availability alters both soil–root and leaf–atmosphere interfaces, and threatens the liquid phase from soil to leaves, thereby reducing the capacity of plants to take up water from the soil. Consequently cavitation occurs, which is a phenomenon by which xylem vessels become air filled and loses their functionality for sap conduction (Barigah *et al.*, 2013; Hsiao and Xu, 2000; McDowell *et al.*, 2008; Mitchell *et al.*, 2013). Mortality may directly occur by hydraulic failure for isohydric seedlings or trees near their maximum height. Although anisohydric plants are

relatively drought-tolerant, they are predisposed to hydraulic failure because they operate with narrower hydraulic safety margins during drought (McDowell et al., 2008) .

Isohydric plants maintain a constant midday leaf water potential (ψ leaf) under drought conditions, by reducing stomatal conductance as necessary to limit transpiration (Sade *et al.*, 2012). Examples of woody plants that displayed isohydric characteristics are wild pistachio (*Pistacia* spp.) (Fardooei, 2001) and African baobab (*Adansonia digitata* L.) (De Smedt *et al.*, 2012; Van den Bilcke *et al.*, 2013).

Anisohydric species, by contrast, allow midday Ψl to decline as soil Ψ declines with drought (McDowell et al., 2008; Sade et al., 2012). In anisohydric behavior, there is a good initial relationship between soil water potential and leaf water potential (Limpus, 2009). Apple (*Malus domestica* Borkh.), eggplant (*Solanum melongena* L.), poplar (*Populus* spp. L.) and cowpea (*Vigna unguiculata* L. Walp.) are classified as anisohydric plants (Limpus, 2009).

2.3 Plant responses to drought stress

Drought stress is affected by climatic, edaphic and agronomic factors. The susceptibility of plants to drought stress varies with stress intensity, factors that accompany stress, the nature of the plant species, and their developmental stages (Demirevska *et al.*, 2009). Drought stress influences plant growth and yield, membrane integrity, pigment content, osmotic adjustment, water relations and photosynthetic activity (Benjamin and Nielsen, 2006; Praba *et al.*, 2009). According to Anjum *et al.* (2011), these effects can be divided in three main categories:

2.3.1 Effect of drought stress on morphological plant characteristics

Plants living in arid and semi-arid regions tend to have special morphological adaptations to reduce water losses. Plant leaves tend to develop xeromorphic characteristics when they grow under dry conditions, in order to prevent excessive water loss.

Van Damme (1990) mentioned morphological adaptations at leaf level tend to reduce transpiration. The latter effect can be obtained through a lowering of the transpiring leaf surface area or by changes at stomatal level. Most important morphological features that can be affected by water deficit are described hereafter.

2.3.1.1 Leaf characteristics

Water availability is an important factor affecting leaf dimension, leaf area, leaf number and leaf coating of plants. The development of an optimal leaf area is important in relation to photosynthesis and dry matter yield. Water deficiency stresses mostly reduce leaf expansion. Consequently, the total leaf area of the plant is reduced (Farooq et al., 2009b; Yang *et al.*, 2004). Water deficit stimulates leaf abscission and leaf wax deposition but decreases leaf area and above ground plant growth (Fulda *et al.*, 2011).

Environmental stresses such as drought stress have important effects on leaf traits (Dong and Zhang, 2000). The leaf is the organ most responsive to environmental conditions; its structure reflects the effects of drought stress more clearly than stems or roots (Fardooei, 2001; Liu and Stützel, 2004). Perennial plants in arid and semi-arid areas tend to have smaller leaves, an adaptation that enables them to conserve water (Ludlow and Ng, 1974).

At plant level, leaf expansion is one of the processes most sensitive to drought stress. In fact, reduced leaf area is probably the more obvious mechanism by which plants and crops will defend themselves against drought stress (Hsiao and Xu, 2000). Water stress greatly suppresses cell expansion and cell growth due to low turgor pressure (Shao *et al.*, 2008).

Under low water availability conditions, leaf area rates significantly decreased in olive (*Olea europaea* L) (Bacelar *et al.*, 2007), peanuts (*Arachis hypogaea*) (Rucker *et al.*, 1995), maize (*Zea mays*) (Khan *et al.*, 2001) and *Sabina vulgaris* (Dong and Zhang, 2000).

Van den Bilcke et al. (2013) mentioned that baobab (*Adansonia digitata* L.) seedlings are leafless during most of the dry season, suggesting a drought avoidance strategy. In many plants that are living in semi-arid and arid zones, drought adaptation is achieved by a reduction of the transpiring area through leaf shedding (Orshan *et al.*, 1989). For instance, leaf shedding is a drought stress coping strategy in *Zygophyllum dumosum* (Sundberg, 1985), wild almond (*Prunus* spp.) (Rouhi *et al.*, 2007) and baobab (*Adansonia digitata* L.) (Van den Bilcke et al., 2013). A number of drought resistant plants even shed their leaves several times a year. Each time, shedding is followed by regrowth of new leaves, which are often smaller and more xerophytic than those just shed (Sundberg, 1985) cited in (Fardooei, 2001; Van Damme, 1990). In *Ziziphus* trees, leaf shedding occurred more in trees after rapid stress treatment compared to gradual stress treatment (Arndt *et al.*, 2001).

There is much mucilage in leaf epidermal cells of baobab seedlings that may help in maintaining a high leaf water potential (Ψ_l) in this plant (Van den Bilcke et al., 2013). Leaf mucilaginous substances increased during drought stress in *Brunella grandiflia* (Jeremias,

1966), whereas leaves of *Ziziphus* plants do not show any significant difference exposed to drought stress treatments (Clifford *et al.*, 2002).

Drought tolerance of wild pistachio species can be related to a deep taproot, high water conservation ability through stomatal adjustment, stomatal features, leaf characteristics, and leaf shedding (Fardooei, 2001; Germana, 1996; Spiegel-Roy *et al.*, 1977).

Leaf area was significantly lower for drought-stressed than for well-watered pistachio (*Pistacia vera*) seedlings (Abbaspour *et al.*, 2012b). Drought stress induced a reduction in evaporation and stomatal conductance in *Pistacia vera* and *P. atlantica* (Behboudian *et al.*, 1986; Goldhamer *et al.*, 1985; Sepaskhah and Maftoun, 1982; Spiegel-Roy et al., 1977).

2.3.1.2 Stomata

Most water loss from plants occurs through the stomata whereas most of the carbon dioxide used in photosynthesis enters through the same structures. Thus, stomatal behavior plays a very important role in plant physiology (Kramer and Boyer, 1995). Stomata can occur on the epidermis of all plant organs, except roots. In dry conditions, stomata in mesophytic species are usually smaller and more numerous than those for species growing in a more humid environment (Kramer, 1969). There are different ideas about stomatal density in plants that are adapted to dry conditions, whereas it surely depends on the kind of plant species (C_3, C_4 or CAM). Some authors mentioned that a low stomatal density is typical for these plants whereas others claimed that this parameter increases with drought (Van Damme, 1990).

One of the first responses of almost all plants to severe water deficit is the closure of their stomata to prevent transpiration water loss (Ali et al., 2008; Farooq et al., 2009b; Maroco *et al.*, 1997; Sharkey and Seemann, 1989; Tezara *et al.*, 1999). Stomatal closure is generally accepted to be the main determinant for decreased photosynthesis under mild to moderate drought (Cornic and Massacci, 1996; Yokota *et al.*, 2002). When the amount of available soil water is moderately or severely limiting, one option for plants is to close their stomata (Cornic and Massacci, 1996). Stomatal characteristics, such as density and size, are greatly affected by species type and environmental factors (Dong and Zhang, 2000; Munir *et al.*, 2011). Drought stress increased stomatal density and decreased stomatal size in olive varieties (Guerfel *et al.*, 2009) while, drought stress decreased stomatal density and size in strawberry (Klamkowski and Treder, 2007). Olive plants grown under drought conditions exhibited a significant increase in number of stomata and trichomes in different varieties when compared to well-watered control plants (Bacelar et al., 2007).

Evaluation of epidermal leaf cells of four pistachio rootstocks (*Pistacia vera*, cv. Badami Zarand, Sarakhs, Ghazvini and *Pistacia mutica*) which are grown under three irrigational levels (100%, 65% and 30% ETc) showed that the highest stomatal density and the lowest stomatal length and width values were obtained with severe drought stress (30% ETc) treatment (Arzani et al., 2013).

Several reports suggested that plant metabolic processes are in fact more sensitive to turgor and cell volume than to absolute water potential, with maintenance of inter-molecular distances critical for continued metabolic activity (Clifford *et al.*, 1998; Jones and Corlett, 1992). Maintenance of cell turgor, which is a hydraulically mediated process, plays an important role in regulating the carbon balance of plants. Growth is particularly sensitive to changes in cell turgor and often declines before reductions in leaf photosynthesis in response to drought are observed (Mitchell et al., 2013; Taiz and Zeiger, 2010).

Cell growth is one of the most drought-sensitive physiological processes due to the reduction in turgor pressure (Taiz and Zeiger, 2010). Therefore, any loss in turgor pressure as a consequence of the imbalance in the plant water content could result in reduced growth and even in the total absence of growth under dry environmental conditions.

Under severe water deficiency, cell elongation of higher plants can be inhibited by interruption of water flow from the xylem to the surrounding elongating cells (Farooq *et al.*, 2009a; Nonami, 1998). Under water deficit and as a result of solute accumulation, the osmotic potential of the cell is lowered, which attracts water into the cell and helps with the maintenance of turgor (Farooq et al., 2009a; Taiz and Zeiger, 2010) .

2.3.1.3 Stem elongation and plant height

In vascular plants, one of the main functions of the stem is transferring water and dissolved minerals from roots to other plant parts and vice versa (Solomon *et al.*, 2008). Drought stress adversely affects growth, stem extension, dry mass, and productivity of plants (Anjum et al., 2011; Blum, 2005; Farooq et al., 2009b; Hussain *et al.*, 2008) and causes a higher rate of impairment than any other environmental factor (Cechin *et al.*, 2008; Shao et al., 2009).

Shoot length is considerably depressed due to drought stress in cowpea and soybean (Wien, 1977) and apple rootstocks (Fernandez *et al.*, 1997). Drought stress caused a significant reduction in plant height, leaf area and stem diameter in maize (Khan et al., 2001) and *Dalbergia sissoo* and *D. latifolia* (Ashraf *et al.*, 2004). Plant height decreased with

drought stress in vetch (*Vicia narbonensis*, *V. sativa* and *V. villosa*) species (Haffani *et al.*, 2014) and citrus seedlings (Jaleel et al., 2009; Wu *et al.*, 2008).

Reduction in plant height and leaf area under drought stress may be associated with a decline in cell enlargement and faster leaf senescence in the plant (Bhatt and Srinivasa Rao, 2005; Manivannan *et al.*, 2007; Shao et al., 2009). A significant decrease in stem length of 'Badami' and 'Ghazvini' pistachio was observed with increasing irrigation intervals, while that of 'Sarakhs' was not significantly affected (Tajabadipour *et al.*, 2006). Plant height of different pistachio cultivars was reduced with increasing salinity stress; this reduction was more pronounced in Ohadi and Kaleghochi cultivars under moderate and high salinities (Abbaspour et al., 2012a).

2.3.1.4 Fresh and dry biomass

Plant growth may be evaluated by monitoring fresh or dry biomass increase or elongation (changes in length). Both parameters may depend more directly on osmotic adaptation and on the maintenance of a positive water balance. Drought severely affects plant growth and development with significant reductions in growth rate and biomass accumulation (Bacelar et al., 2007; Maraghni *et al.*, 2011; Sanders and Arndt, 2012; Shao et al., 2008).

In alfalfa (*Medicago sativa*), shoot and root fresh and dry weight was reduced by polyethylene glycol-induced water deficit, while root length was increased (Zeid and Shedeed, 2006).

Shoot and root dry weight of pistachio seedlings were significantly depressed with increasing salinity stress (Bastam et al., 2013; Hajiboland et al., 2014; Karimi *et al.*, 2009). Saadatmand *et al.* (2007) indicated that salinity stress had more negative influence than drought stress on plant height, shoot and root dry weights. With increase in salinity stress, dry weight of shoots decreased in Akbari, Kaleghochi and Ohadi pistachio cultivars (Abbaspour et al., 2012a).

Shoot dry weights were reduced in Badami, Qazvini and Sarakhs pistachio cultivars with increasing irrigation intervals (Tajabadipour et al., 2006). The fresh and dry weight of *Pistacia vera* L. 'Ahmadaghaii' seedlings decreased significantly under drought stress conditions in both with and without silicon treatments in the soil (Habibi and Hajiboland, 2013) .

2.3.1.5 Yield

Water limitation not only reduces growth of leaf and stem expansion but also has a negative impact on yield, e. g. by decreasing fruit size in fruit crops (Sami, 1992). As an example, increasing the irrigation interval of adult pistachio trees to more than 30 days (50, 80 and 110 days) had a negative effect on fresh yield and this was even more pronounced in the case of dry yield (Sedaghati and Hokmabadi, 2015).

As with other tree species, irrigation increases pistachio yield, but with this species it also improves nut quality (percentage of split nuts is higher) and reduces normal, alternate bearing pattern (Goldhamer, 1995; Kanber et al., 1993; Panahi et al., 2002). Goldhamer et al. (1985) mentioned that severe water stresses (0-25 % evapotranspiration=ET) regimes showed significant decreasing effects on vegetative growth and crop yield in adult pistachio trees.

Water deficiency on mature pistachio trees during rapid kernel growth to harvest, significantly reduced nut size, shell splitting and yield (Goldhamer and Beede, 2004). Regulated deficit irrigation of trees showed total yield and percentage of split nuts similar to those of the controls, even though they received around 20% less water (Gijon *et al.*, 2009).

2.3.2 Effect of drought stress on physiological characteristics

2.3.2.1 Photosynthesis

Net photosynthesis (*Pn* in μmol CO_2 m^{-2} s^{-1}) is the difference between the total amount of photosynthesis and the sum of the respiration rates (i.e. photorespiration and dark respiration). Transpiration (E in mmol H_2O m^{-2} s^{-1}) is the process of water movement through a plant and its evaporation from aerial parts, such as leaves, stems and flowers. Stomatal conductance (g_s in mol H_2O m^{-2} s^{-1}) expresses the rate at which carbon dioxide (CO_2) rate enters, or water vapor exits through the stomata.

Drought can induce significant reduction of net photosynthesis and productivity in crops. Plant physiological responses such as photosynthesis and transpiration depend on the timing, severity, and length of the drought event (Anjum et al., 2011; Chaves *et al.*, 2002; Praba et al., 2009). Plants response to water stress by stomatal closure (Flexas *et al.*, 2002; Lawlor, 2002). As a result, photosynthetic assimilation is unavoidably reduced due to decreased CO_2 concentrations at chloroplast level (Cornic, 1994a). Drought may also reduce photosynthesis by other mechanisms such as loss of leaf turgor (McDowell et al., 2008) and leaf shedding (Taiz and Zeiger, 2010). Turgor pressure maintenance allows stomata to remain open, photosynthesis to continue and growth to be uninterrupted (Pessarakli, 1994). Furthermore,

under progressive drought stress, the electron transport will be impaired followed by an enhanced production of reactive oxygen species (ROS) (Cornic and Fresneau, 2002; Cruz de Carvalho, 2008; Gnaana Saraswathi and Paliwal, 2011).

Drought stress treatments decreased net photosynthetic rate (*Pn*), transpiration rate (E), and stomatal conductance (g_s) of apple (*Malus domestica*) trees compared to a well-watered control treatment (Liu *et al.*, 2012). Under drought, net photosynthesis can reduce due to both stomatal (decrease in stomatal conductance and reduce intercellular CO_2) and non-stomatal (mesophyll resistance= biochemical processes) limitations (Behboudian et al., 1986; Fardooei, 2001; Flexas *et al.*, 2009). Decreased stomatal aperture limits CO_2 influx resulting in a decline in the rate of photosynthesis under stomatal limitations. Stomatal limitations are often thought to be the short term response to drought stress, whereas non-stomatal effects are usually considered to be more important during longer and more severe drought stress events (Farooq et al., 2009a). Decreased synthesis and altered activities of essential enzymes and photosynthetic pigments, impaired ATP synthesis, photorespiration and heavy oxidative load are among the major non-stomatal limitations of carbon fixation (Farooq et al., 2009a; Sanders and Arndt, 2012) . Ogren and Oquist (1985) reported that under low drought stress, *Pn* is limited by stomatal closure, whereas under high drought stress non-stomatal factors prevail. Photosynthesis reduction under drought stress will impair primary nitrogen assimilation (Bauer *et al.*, 1997; Xu and Zhou, 2006).

Photosynthetic rates and stomatal conductance of *Citrus unshiu* trees were significantly reduced with increasing dehydration (Yakushiji *et al.*, 1998). Klamkowski and Treder (2007) indicated a decrease in *Pn*, E, g_s and intercellular CO_2 (C_i) concentration under water deficit in stressed plants compared to well-watered plants in strawberry (*Fragaria ×ananassa*).

Ranjbarfordoei *et al.* (2000) reported that net photosynthetic rates were more reduced for *P. mutica* than for *P. khinjuk* under increasing osmotic drought stress, indicating a higher tolerance to drought of *P. khinjuk*. Filella *et al.* (1998) mentioned watered plants had higher *Pn* and g_s than non-watered plants in *Phillyrea latifolia* (Oleaceae), *Pistacia lentiscus* (Anacardiaceae) and *Quercus ilex* (Fagaceae).

Photosynthesis in *Pistacia vera* was lowered with decreasing leaf water potential (Behboudian et al., 1986). Bagheri et al. (2011) found that Qazvini pistachio cultivar was more tolerant to drought stress than Badami as it maintained a higher photosynthetic activity under drought. Leaf transpiration rate of *P. atlantica* decreased under severe drought stress compared to moderate water deficit (Fayyaz *et al.*, 2013). These results confirm the two-step

drought avoidance mechanisms of water spending and saving in *P. atlantica* as reported for *P. terebinthus* and *P. lentiscus* (Chirino *et al.*, 2011; Vilagrosa *et al.*, 2003).

Drought stress decreased net assimilation rate in pistachio, while this reduction was alleviated by silicon application in the soil, accompanied by an increase in stomatal conductance (Habibi and Hajiboland, 2013). Silicon (Si) application to crops has been reported to enhance their tolerance of water stress (Ma, 2004). Silicon causes an improvement of water usage efficiency and stimulates enzymatic and non-enzymatic antioxidative defense systems (Hattori *et al.*, 2005; Liang *et al.*, 2007). An increase in the production of antioxidants and a decline of reactive oxygen species (ROS) generation mediated by Si causes a reduction of photo-oxidative damage, maintenance of chloroplast membrane integrity and thus enhancement of plant drought tolerance (Waraich *et al.*, 2011). Photosynthetic rate, stomatal conductance, and transpiration also could be affected by the application of salicylic acid in maize (Zea mays L.) (Khan et al., 2001).

2.3.2.2 Photosynthesis pigments

Thylakoid membranes contain several kinds of pigments, which are substances that absorb visible light. The main pigment of photosynthesis is chlorophyll (Chl), which absorbs light in the blue and red regions of the visible spectrum. Another pigment is carotenoids (Car) that absorbs different wavelengths of light than chlorophyll (Solomon et al., 2008). Carotenoids absorbs light in the near ultraviolet (UV) as well as in the visible region. In photosynthetic organisms, carotenoids are bound, together with chlorophylls, to proteins and participate to light harvesting. An essential function of carotenoids in photosynthesis is to prevent harmful photooxidative reactions related to the presence of oxygen (Pessarakli, 1994). The light absorbed by the carotenoids is transferred to chlorophyll for photosynthesis (Taiz and Zeiger, 2010).

Water stress changes in the ratio of Chl a, Chl b and Car (Anjum et al., 2011; Farooq et al., 2009b). However, Car has additional roles and partially helps the plants to withstand adversaries of drought (Jaleel et al., 2009).

Rouhi (2007) reported that exposure of three wild almond species (*Prunus dulcis*, *P. lycioides* and *P. scoparia*) to osmotic drought stress, led to increased Chl *a* concentrations and decreased Car contents. Drought stress treatment decreased total Chl content in apple (*Malus domestica*) trees (Liu et al., 2012), *Albizia lebbeck* (lebbeck tree) and *Cassia siamea* (cassia tree) seedlings (Saraswathi and Paliwal, 2011) compared to corresponding control plants.

Ranjbarfordoei *et al.* (2002) reported that exposure of two pistachio species (*P. khinjuk* and *P. mutica*) to osmotic drought stress led to lower chlorophyll *a* and *b* contents. Arbuscular mycorrhiza (AM) colonization and plant growth and total chlorophyll were higher for well-watered than for drought stressed plants in pistachio Badami (Abbaspour et al., 2011).

Impact of salinity on reduction of chlorophyll content was significant in all three pistachio cultivars i.e. Akbari, Kaleghochi and Ohadi tested by Abbaspour et al. (2012a). Chlorophyll pigment concentrations were increased with mild drought stress compared with control, and then decreased with increasing stress intensity in pistachio rootstocks. Carotenoid content increased significantly in all drought-stress levels (Bagheri et al., 2011). Bastam et al. (2013) reported salicylic acid (SA) increased relative leaf chlorophyll content and photosynthetic capacity as compared with control in salt stress conditions. The relatively higher leaf chlorophyll content values of SA-treated pistachio leaves may be related to the influence of salicylic acid on endogenous cytokinin contents. SA-treated plants synthesized more cytokinin (Sakhabutdinova *et al.*, 2003) which, in turn, enhanced chloroplast differentiation, chlorophyll biosynthesis, and prevented chlorophyll degradation (Fletcher and Arnold, 1986).

2.3.2.3 Chlorophyll fluorescence

The principle of chlorophyll fluorescence measurements is based on the fact that a fraction of the absorbed photon energy is released as a photon with a lower energy content (higher wavelength) (Maxwell and Johnson, 2000). For example, Chl a absorbs photons in the blue and red regions of the spectrum whereas emission is in the red region of the spectrum. This phenomenon is called chlorophyll fluorescence (Hall and Rao, 1999; Maxwell and Johnson, 2000). Chlorophyll fluorescence emission provides a fast indicator of the primary photochemistry of photosynthesis and reflects the primary processes that takes place in the chloroplast such as light absorption, excitation of energy transfer and the photochemical reaction in PSII (DeEll *et al.*, 1999). The perturbations of photosynthetic metabolism which are induced by drought stress will significantly modify the fluorescence emission and kinetic characteristics of plants. Therefore, chlorophyll fluorescence provides useful information about leaf photosynthetic performance under drought stress (Baker and Rosenqvist, 2004).

There are different types of apparatus to measure chlorophyll fluorescence. In recent years, this technique is used as a tool for studying many aspects of plant physiology related to photosynthesis (DeEll et al., 1999; Ganago, 1998; Maxwell and Johnson, 2000; Oliveira and Peñuelas, 2000). It is a rapid, powerful and non-destructive technique, with the potential to

identify photosynthetic tolerance to drought stresses in plants and can be used as a predictor of stress injury in plant physiology before damage to plants is visible (Baker and Rosenqvist, 2004; DeEll et al., 1999; Krause and Weis, 1991; Maxwell and Johnson, 2000).

Pulse-Amplitude-Modulated (PAM) chlorophyll fluorescence measurements are widely used as a simple, rapid, and non-invasive method to assess the physiological state of higher plants and algae (Juneau *et al.*, 2005). Upon irradiation of a dark-adapted plant, the fluorescence signal rises quickly and then decreases to reach a steady-state level (Figure 2.1). This decrease, or quenching, of the fluorescence yield is due to both increased photochemistry and radiation-less deactivation processes (Roháček, 2002). The measurement by PAM is initiated by switching on the measuring light, giving a measure of the F_0 (minimal) level of fluorescence. A saturating flash of light is then applied, allowing the measurement of F_m (fluorescence maximum) in the dark-adapted state ($F^0{}_m$). Next, an actinic light is applied and, at appropriate intervals, further saturating flashes are applied. From each of these, a value for F'_m, the fluorescence maximum in the light, can be measured. The steady-state value of fluorescence immediately prior to the flash is termed F_t. There after a flash, removal of actinic light (preferably whilst simultaneously giving a far-red light) allows measurement of F'_0 (zero level fluorescence in the light) (Maxwell and Johnson, 2000).

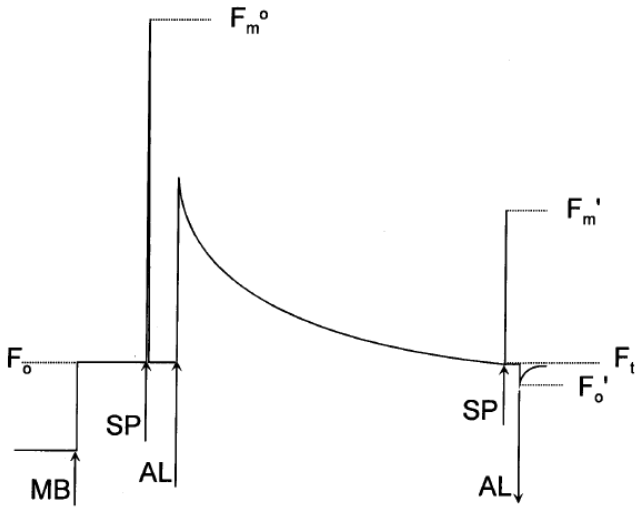


Figure 2.1: Sequence of a typical fluorescence trace (Maxwell and Johnson, 2000).

The photochemical quenching (qP) provides information on the redox state of QA (the primary electron acceptor of PSII); the non-photochemical quenching (NPQ) reflects the loss of excess energy through heat dissipation (DeEll et al., 1999).

Well-known fluorescence parameters for assessing the functional integrity of PSII include:

*. **F_v/F_m (maximum quantum yield of PSII photochemistry after dark adaptation);**

Ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) is used as maximal quantum yield of PSII photochemistry (F_v/F_m). The chlorophyll fluorescence ratio (F_v/F_m) was calculated as:

$F_v/F_m = (F_m - F_0) / F_m$

Where F_m and F_0 are the maximum and minimum (basal) chlorophyll fluorescence yields of dark-adapted leaves respectively. Also, F_v is the maximum variable chlorophyll fluorescence yield in the dark-adapted state. F_v/F_m provides information about the underlying processes. A change in F_v/F_m is due to a change in the efficiency of non-photochemical quenching. Dark adapted values of F_v/F_m reflect the potential quantum efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance. The F_v/F_m in plants which were grown in suitable conditions is around 0.8. Values lower than 0.8, will be seen when the plant has been exposed to stress, hence, indicating in particular the phenomenon of photoinhibition (Maxwell and Johnson, 2000; Roháček, 2002). Therefore, in order to demonstrate the tolerance of plants to water stress, F_v/F_m is a very good parameter.

*. **YII (effective quantum yield of PSII electron transport);** this is calculated as:

$YII = (F'_m - F_s) / F'_m$

Where F'_m is the maximum chlorophyll fluorescence in leaves submitted to ambient light and F_s is the steady fluorescence in leaves acclimated to ambient light. This parameter (YII) measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry. Therefore, it can give a measuring rate of linear electron transport and so an indication of overall photosynthesis (Maxwell and Johnson, 2000; Roháček, 2002).

***. qP (photochemical quenching)** is another widely used fluorescence parameter measuring photochemistry, and it is calculated as:

$qP = (F'_m - F_s) / (F'_m - F'_0)$

Where F'_m is the maximum fluorescence in leaves submitted to ambient light, F_s is the steady fluorescence in leaves acclimated to ambient light and F'_0 is initial fluorescence in leaves submitted to ambient light. Although qP seems superficially very similar to YII, the significance of this parameter is somewhat different. YII is the proportion of absorbed energy being used in photochemistry, while qP gives an indication of the proportion of photosystem II (PSII) reaction centers that are open (Maxwell and Johnson, 2000; Roháček, 2002). Photochemical quenching (qP) indicates the ability of PSII to transfer electrons to various

acceptors within the chloroplast. A change in qP is due to closure of reaction centers, resulting from a saturation of photosynthesis by light. qP values may vary between 1and 0 under control and stress conditions. When qP is high, it shows reaction centers are open and electron transport will go easily. When qP is low and decreased compared to control plants, it indicates that relative closure of reaction centers and a decreased electron transport.

***. NPQ (non-photochemical quenching of chlorophyll fluorescence);**

The most straightforward way of quantifying non-photochemical quenching is by measuring the ratio of a change in F_m to the final value of F_m:

$(F^0_m - F'_m) / F'_m$

Non-photochemical quenching is one of the mechanisms that prevents or alleviate damage to the photosynthetic apparatus. In this mechanism, excess radiation energy is dissipated as heat in the light harvesting antenna of PSII (Müller *et al.*, 2001). NPQ is related to heat dissipation and lies on a scale from zero till infinity. In a typical plant, values might be expected in the range 0.5–3.5 at saturating light intensities; however, this varies markedly among species and also varies on the previous history of the plant (Maxwell and Johnson, 2000).

Since YII is the effective quantum yield of PSII photochemistry, it can be used to calculate linear electron transport rate and, therefore, overall photosynthetic capacity in vivo (Genty *et al.*, 1989). The electron transport rate (ETR) was calculated as YII × PAR × 0.84 × 0.5, where the absorbed photon energy (PAR= Photosynthetic active radiation) is assumed to be equally distributed between PSI and PSII and 0.84 is the assumed light absorptance of the leaf (Maxwell and Johnson, 2000).

Based on the type of physiological stress and its effect on the photosynthesis pathway, one or all fluorescence parameters could be critical and sensitive indicators (DeEll et al., 1999; Kooten and Snel, 1990; Maxwell and Johnson, 2000).

Chlorophyll fluorescence features (F_v/F_m and qP) of wheat (*Triticum aestivum*) under drought (Hassan, 2006) and barley (*Hordeum vulgare*) under saline stress conditions (Jiang *et al.*, 2006; Li *et al.*, 2006) significantly decreased compared to control plants. In strawberry *(Fragaria ×ananassa)* a gradual reduction in photochemical quenching (qP) and quantum efficiency (YII) was observed under drought stress while non-photochemical quenching (NPQ) increased (Razavi *et al.*, 2008).

Salicylic acid treatment increased the maximum quantum yield of PSII (F_v/F_m) of pistachio seedlings significantly, compared with control plants under salt stress conditions (Bastam et al., 2013), indicating that salicylic acid reduced salt-induced photoinhibition by

protecting photosystem II. Salicylic acid is known as an important factor in the plant defense system during pathogen attack. It is also involved with osmotic stress and can amplify the effects of osmotic stress by increasing the ROS production during photosynthesis; this ROS acts as a signal which might improve the systemic resistance (Bartoli et al. 2012).

Ranjbarfordoei et al. (2002) reported that the heat dissipation (NPQ) only significantly increased at the highest level of drought stress (-1.55 MPa) in *Pistacia khinjuk* and *P. mutica*. F_v/F_m was adversely affected by drought stress in pistachio cultivar Qazvini while in Badami cultivar, increasing drought-stress intensity had no significant effect on this parameter (Bagheri et al., 2011). The soil application of silicon in pistachio seedlings showed that F_v/F_m improved under drought stress compared with non-treated silicon seedlings. Providing optimum CO_2 concentration for carbon reactions may prevent photo-inhibition, which was clearly reflected in the significantly higher F_v/F_m values in the presence of silicon in drought-stressed pistachio plants (Habibi and Hajiboland, 2013).

2.3.2.4 Water relations

Plant–water relations concern how plants control the hydration of their cells, including the collection of water from the soil, its transport within the plant and its loss by transpiration from the leaves.

It is well-known that most (95% or more) of the water absorbed from the soil by the roots of a tree is not retained in the tree, but evaporates through stomatal pores located on the leaf surface; a process which is referred to as transpiration (Ridge, 2002). Each day, water must move from the roots to the leaves to replace the quantity lost by transpiration. Furthermore, this flow of water also promotes the transport to leaves of mineral nutrients absorbed by the roots.

Soil water enters the root through small root hairs that are located on the epidermal cells of the roots. It moves radially across the root cortex to the central cylinder which includes the vascular tissue. The water conducting elements of the vascular tissue are primarily the non-living and heavily thickened and lignified tracheids and xylem vessels. They act as a network of tubes connecting one to another, tubes which the water can ascent from the roots up to the small veins in the leaves. At the veins' ends, the water is released from the xylem. After crossing some mesophyll cells and/or cell walls, it reaches the substomatal cavity (an intercellular air space) from where it finally diffuses through the stomatal pores into the atmosphere as water vapor. The ascent of liquid water through the tree induced by this transpiration process is defined as sap flow (or transpiration stream) (Steppe, 2004).

The water status of plants is usually expressed as water potential. The driving force which governs the water transport in trees originates from the difference in total water potential whereby water will flow spontaneously from a region of higher water potential (less negative) to one of a lower water potential (more negative).

According to the different forces which can act on water molecules, the total water potential ($\Psi_{w,}$ MPa) can be written as (Nobel, 1999):

$\Psi_w = \Psi_P + \Psi_m + \Psi_s + \Psi_g$

In this equation, Ψ_p is the hydrostatic potential or pressure potential (MPa), Ψ_m is the matrix potential (MPa), Ψ_s is the osmotic or solute potential (MPa) and Ψ_g is the gravitational potential (MPa). The water potential is zero in pure and free water at standard atmospheric pressure, and at a temperature of 298 K (25° C) (Passioura, 2010).

The components of the total water potential can have a positive and/or negative value, depending on the type of physical and chemical forces which interfere with the mobility of water molecules in the system (Steppe, 2004).

The pressure potential (Ψ_p), referring to the physical pressure exerted on water in the system, can be positive or negative. For example, water in the turgid root cortical cells or leaf mesophyll cells are under positive turgor pressure that exerted pressure against the cell walls ($\Psi_p > 0$), whereas water in the dead xylem vessels of a transpiring plant is under tension or suction (negative hydrostatic pressure; $\Psi_p < 0$). The positive hydrostatic pressure within cells is the pressure which is referred to as turgor pressure. The value of Ψ_p can also be negative, as is the case in the xylem and in the walls between cells, where a tension, or negative hydrostatic pressure, can develop. Negative pressures outside cells are very important in moving water long distances through the plant (Munns *et al.*, 2000; Taiz and Zeiger, 2010).

The matrix potential (Ψ_m), dealing explicitly with interactions occurring at the numerous air-water interfaces of a cell wall, is always negative. Large negative pressures on water in cell wall interstices arise because of capillary effects due to the attraction between water and the hydrophilic cell wall surface at an air-water interface.

Osmotic potential (Ψs) represents the effect of dissolved solutes on water potential. Solutes reduce the free energy of water by diluting the water (Lichtfouse *et al.*, 2009; Taiz and Zeiger, 2010).

Gravity causes water to move downward unless the force of gravity is opposed by an equal and opposite force. The term Ψg depends on the height (h) of the water above the reference-state water, the density of water, and the acceleration due to gravity (g) (Taiz and Zeiger, 2010).

Cell growth, photosynthesis, and crop productivity are all strongly influenced by water potential and its components (Comstock and Mencuccini, 1998; Taiz and Zeiger, 2010). Like the body temperature of humans, water potential is a good overall indicator of plant health (Taiz and Zeiger, 2010).

Plant water relations during drought stress play a critical role in the activation or regulation of different metabolic defense mechanisms (Sánchez-Rodríguez *et al.*, 2010). Plants continuously absorb and lose water during their life (Taiz and Zeiger, 2010). Important characteristics to evaluate the plant water relations are as follow (Farooq et al., 2009b):

2.3.2.4.1 Relative Water Content (RWC)

RWC is the water content per unit leaf mass that is related to its fully hydrated or fully turgid state. It indicates the hydration state of the leaf. RWC is calculated as RWC = (fresh weight -dry weight)/ (turgid weight -dry weight) (Flower and Ludlow, 1986). Relative water content is often used as an index to measure dehydration tolerance (Anjum et al., 2011; Blum, 1996; Flower and Ludlow, 1986).

Drought tolerance of a plant is related to its ability to maintain high RWC in their leaves under water deficit conditions (Bohnert and Jensen, 1996; Rampino *et al.*, 2006; Suprunova *et al.*, 2004). Relative water content is related to water uptake by the roots and water loss by transpiration. When plants are subjected to drought stress treatments, relative water content decreased in wide variety of plants (Anjum et al., 2011; Nayyar and Walia, 2003).

High relative water content (RWC) was maintained by stomata closure and leaf area reduction under water deficit conditions in cowpea (*Vigna unguiculata*) (Anyia and Herzog, 2004). Relative water content and leaf water potential (ψ_w) of olive (*Olea europaea* L.) trees decreased with increasing drought stress levels (Boussadia *et al.*, 2008). Relative water content, leaf water potential and transpiration were decreased under drought stress on wheat (*Triticum aestivum*) and rice (*Oryza sativa* L.) (Siddique *et al.*, 2000) and *Hibiscus rosa-sinensis* (Egilla *et al.*, 2005).

In pistachio (*P. vera*), RWC and water use efficiency (WUE) values vary with different fruit growth stages and cultivars (Sajjadinia *et al.*, 2010). The highest RWC among pistachio cultivars were in Ohadi, Kaleghochi and Ahmadaghaii cultivars (Fotohi Ghazvini *et al.*, 2007). Under drought conditions, silicon (Si) supplementation improved plant growth and increased relative water content in pistachio (Habibi and Hajiboland, 2013).

2.3.2.4.2 Leaf Water Potential (LWP)

The leaf water potential has been the primary index of the crop water status. There are few techniques for measuring leaf water potential that are truly suitable for field work (Knipling, 1967; Turner, 1981). The pressure chamber is the equipment widely used to measure the water potential (Jarvis, 1976; Turner, 1981) The pressure chamber (Figure 2.2) is a device for applying air pressure to a leaf (or small shoot), where most of the leaf is inside the chamber but a small part of the leaf stem (the petiole) is exposed to the outside of the chamber through a seal. The amount of pressure that it takes to cause water to appear at the petiole tells you how much tension the leaf is experiencing on its water: a high value of pressure means a high value of tension and a high degree of water stress. The units of pressure most commonly used are the Bar and the Mega Pascal (1 MPa = 10 bars).

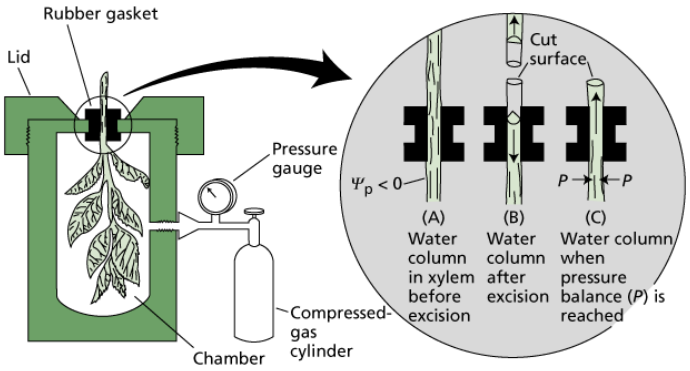


Figure 2.2: The pressure chamber method for measuring plant leaf water potential.

The thermocouple psychrometer (Figure 2.3) is another important and standard instrument for the measurement of the total water potential (Pessarakli, 1994; Turner, 1981). It can measure the water status of any plant part as well as soil or any substance containing water. The technique works over the entire range of water contents and, because it measures conditions in the gas phase, it does not require a continuous liquid phase for the measurement. The only requirement is that water be able to evaporate from the sample to the air. The method uses only a small sample which can be important for repeated measurements in the same plant or soil.

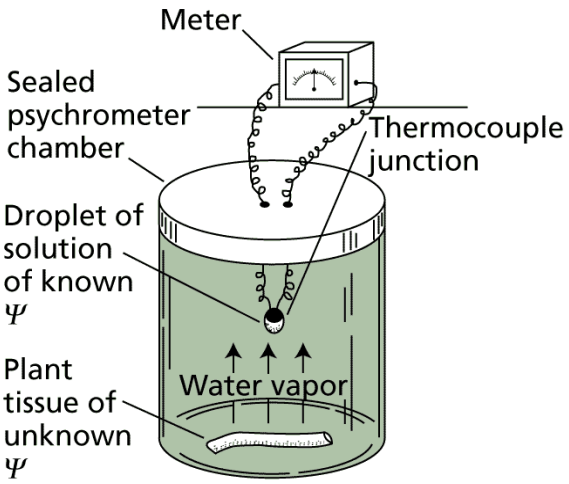


Figure 2.3: The thermocouple psychrometer instrument to measure the water potential of a plant tissue.

The dye (density) method is another useful procedure for measuring leaf water potential. It is simple, inexpensive, and suitable for both laboratory and field work. In this method, leaves are immersed in a graded series of solutions, and the solution which neither gains nor loses water is assumed to have a water potential equal to that of the leaf (Knipling, 1967). There are three important indicators of leaf water potentials that are described as follows;

2.3.2.4.2.1 Pre-dawn leaf water potential (PLWP)

The pre-dawn leaf water potential is obtained by measuring leaf water potential before sunrise, when the stomata of the plant are closed and when the plant has been able to equilibrate its water potential with the most humid layer of the soil (Deloire and Heyns, 2011; Pessarakli, 1994) .

2.3.2.4.2.2 Leaf water potential (LWP)

The leaf water potential indicates the plant water status during the day. It is a parameter which enables the measurement of a short term hydric plant response in reaction to a change in equilibrium in root water absorption and leaf transpiration (Deloire and Heyns, 2011; McDowell et al., 2008; Munns et al., 2000).

2.3.2.4.2.3 Stem water potential (SWP)

The stem water potential is measured on leaves which are bagged with both a plastic sheet and aluminum foil at least 30 minutes before measurement. Bagging of the leaves prevents

transpiration and their water potential reaches an equilibrium with the water potential in the stems. Stem water potential is generally measured between 11h00 – 15h00, when values reach to the minimum (Deloire and Heyns, 2011).

A decrease in leaf water potential suppresses photosynthetic activity in plants so that the rate of CO_2 fixation is reduced. Reduction in leaf water potential affects the activity of the electron-transport chain in chloroplasts, quantum yield of O_2 evolution and activity of ATP-synthesis which is ultimately reflected in growth and yield (Bartoli *et al.*, 2005; Nogués *et al.*, 2002; Ogren and Oquist, 1985). Decreasing leaf water potential (Ψ_l) with increasing drought stress is known as a mechanism of plants to survive drought stress conditions (Anjum et al., 2011; Ashraf et al., 2004; Naor, 1999; Rouhi et al., 2007). Drought-tolerant plants are expected to reach lower leaf osmotic potentials than drought-sensitive ones when exposed to water deficit conditions (Anjum et al., 2011; Porcel and Ruiz-Lozano, 2004; Rouhi et al., 2007). Reductions in leaf water potential will reduce stomatal conductance and stomata will close. This simultaneously restricts the entry of CO_2 into the leaf, reducing photosynthesis (Baker and Rosenqvist, 2004). Isohydric plants maintain a constant midday leaf water potential (Ψ) when water is abundant, as well as under water deficiency conditions, by reducing stomatal conductance (g_s) to limit transpiration. In anisohydric plants, leaf water potential decreases with a decline in soil water potential under water shortage condition (Sade and Moshelion, 2014).

Drought stress decreased the leaf water potential in strawberry *(Fragaria ×ananassa)* (Klamkowski and Treder, 2007; Razavi et al., 2008). In faba bean (*Vicia faba* L.) however, leaf water potential was not the defining feature of drought tolerance (Mooney, 1980).

Germana (1996) found that midday xylem water potentials of adult pistachio plants did not drop below -0.5 MPa under drought because of their deep, expanded root systems. Behboudian et al. (1986) mentioned that photosynthesis rate of *P. vera* declined when leaf water potential decreased. Leaf water potential and leaf relative water content (RWC) of salt and polyethylene glycol (PEG) treated plants decreased over time in pistachio and *P. vera* Badami plants showed significantly lower potentials than the other cultivars tested (Panahi, 2009). Leaf water potential and turgor potential unexpectedly increased by an increase in watering frequency in three pistachio cultivars (Badami, Ghazvini, and Sarakhs). When pistachio leaf water potential dropped to −0.37 MPa, a turgor potential of approximately 0.90 MPa was maintained. Lowering of water potential to such a degree would probably result in the loss of turgor in many other fruit tree species. However, under increasing water stress, a

significant decline in osmotic potential values was observed for all pistachio cultivars (Tajabadipour et al., 2006).

2.3.2.4.3 Water Use Efficiency (WUE)

Water use efficiency is defined in many ways, depending on the scale of measurement and the main purpose of the study. For plant physiologists, the basic unit of reduction could be moles of carbon gained in photosynthesis (A) in relation to water used in transpiration (E) or to stomatal conductance (g_s), which permits the calculation of instantaneous WUE leaf (A/E) or intrinsic WUE leaf (A/g_s) (Monclus *et al.*, 2006; Ricardo, 2012). Plants with higher WUE are expected to better tolerate water-deficit conditions (Bartels and Sunkar, 2005; Larcher, 2003; Razavi, 2012; Taiz and Zeiger, 2010).

Drought stress considerably increased water-use efficiency compared to well-watered plants in wheat (Abbate *et al.*, 2004), although it had the opposite effect in maize (*Zea mays*) (Anjum et al., 2011). Intrinsic water use efficiency, increased for *Prunus dulcis* with increasing drought stress, while a different pattern was observed for *P. lycioides* and *P. scoparia*, indicating non-stomatal processes prevail over stomatal limitations of the assimilation process (Rouhi et al., 2007).

Ranjbarfordoei et al. (2002) reported that leaf water potential, leaf osmotic potential, net photosynthetic rate, stomatal conductance and water use efficiency in two wild pistachio species (*Pistacia khinjuk* and *P. mutica*) decreased as osmotic potential of the nutrient solution decreased. Net assimilation rate (A), transpiration rate (E) and stomatal conductance (g_s) all were inhibited by exposure of pistachio plants to drought stress. Under silicon (Si) application, water use efficiency (WUE) remained unchanged. Although application of silicon on pistachio plants did not have a conservative effect on water loss because of elevated stomatal conductance, Si might help plants to maintain their water balance despite greater water loss, which might be due to a higher water uptake by stimulation of deeper and stronger root system (Ahmed *et al.*, 2011; Habibi and Hajiboland, 2013). It has been demonstrated that the application of Si improves the water status of stressed plants (Shen *et al.*, 2010). Tavallali *et al.* (2009) mentioned that water use efficiency decreased under salinity stress and zinc deficiency due to a decline in g_s.

2.3.2.5 Osmolyte accumulation

Under drought, leaf turgor maintenance may be achieved by osmotic adjustment the accumulation of proline, sucrose, soluble carbohydrates, glycine betaine, and other solutes in

cytoplasm that improve water uptake from a drying soil (Anjum et al., 2011; Rhodes and Samaras, 1994; Serraj and Sinclair, 2002). These compatible solutes not only help to maintain turgor pressure but also to protect cell enzymes and macro molecules from the damaging effects of reactive oxygen species (ROS) (Farooq et al., 2009b; Ricardo, 2012).

Osmotic stress in plants can result from many environmental conditions, including drought, saline soil, low temperature, pathogen attack and mechanical wounding. These undesirable conditions interfere with plant growth and development, leading to the reduction in crop productivity (Luan, 2002). The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity or drought, often accompanied by a significant decrease in net CO_2 assimilation rate (Popp and Smirnoff, 1995; Murakeozy et al., 2003).

Osmotic adjustment is a mechanism to maintain water relations under osmotic stress (Farooq et al., 2009b), a key adaptation of plants to minimize the effects of drought-induced damage (Blum, 2005) and helps plants under drought in two ways: it (1) helps maintain leaf turgor to improve stomatal conductance for efficient intake of CO_2 (Kiani *et al.*, 2007), and (2) promotes the root's ability to take up more water (Chimenti *et al.*, 2006). Effective control of water loss through stomatal closure, leaf drop or water uptake by enhanced root growth can improve plant water status. Osmotic adjustment through the active accumulation of solutes in the cell sap, rather than through passive solute accumulation, resulting from reduced cell volume can also contribute to turgor maintenance, and this is a precondition for continued growth during drought (Clifford et al., 1998; Hsiao *et al.*, 1976).

2.3.2.5.1 Proline

Proline is a multifunctional metabolite and functions as an osmolyte in osmotic adjustment. It also acts as a stabilizer of sub-cellular structures; non-enzymatic antioxidant; protect proteins and DNA by scavenging ROS; an energy sink; a source for carbon and nitrogen and a stress-related signal under drought stress (Bartels and Sunkar, 2005; Matysik *et al.*, 2002; Nanjo *et al.*, 1999; Zhu, 2002).

There are several reports that underline the significant effects of proline in stress tolerance. Proline accumulation is a very common response of plants exposed to a water-deficit stress in order to reduce cell injury (Anjum et al., 2011; Pérez-Pérez *et al.*, 2009; Singh *et al.*, 2000; Wahid *et al.*, 2007). Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants (Krasensky and Jonak, 2012; Szabados and Savoure, 2010).

Proline contents increased under drought stress in pea (*Pisum sativum*) (Alexieva *et al.*, 2001), petunia (*Petunia inflata*) (Yamada *et al.*, 2005), canola (*Brassica napus* L.) (Mirzaei *et al.*, 2013), maize (*Zea mays*) (Mohammadkhani and Heidari, 2008), jujube (*Ziziphus rotundifolia*) (Arndt et al., 2001) and strawberry (*Fragaria ananasa*) (Razavi, 2012; Zhang and Archbold, 1993). Fulda et al. (2011) reported the accumulation of inositol, glucose, proline, fructose and sucrose in leaves of drought-stressed sunflower (*Helianthus annuus*). Total free amino acids such as; proline, glutamic acid, glycine and lysine changed under severe drought stress. Proline, which is often used as stress indicator, was not related to drought stress in potted apple (*Malus domestica*) trees (Šircelj *et al.*, 2005).

Drought stress increased proline content of arbuscular mycorrhiza (AM)-inoculated and non-AM-inoculated pistachio plants compared with well-watered conditions (Abbaspour et al., 2011). Free proline increased significantly with increasing salinity in leaves of *Pistacia atlantica* subsp. *atlantica* (Benhassaini *et al.*, 2012) and *P. vera* cv. Ghazvini rootstock (Hokmabadi *et al.*, 2005).

2.3.2.5.2 Carbohydrate

During drought stress, soluble sugars accumulate and starch levels decrease in leaves of many plant species (Ingram and Bartels, 1996; Kempa *et al.*, 2008), despite the reduction in carbon fixation during drought stress due to both stomatal closure and down-regulation of the Calvin cycle (Bartels and Sunkar, 2005; Xue *et al.*, 2008).

In general, sugars can function as osmolytes to preserve cell turgor whereas they also have the capacity to protect membranes and proteins from stress damage (Black and Pritchard, 2002; Krasensky and Jonak, 2012; Roitsch and González, 2004). Increased sucrose concentration in drought-stressed leaves were accompanied by decreases in leaf starch concentration in strawberry (*Fragaria ananasa*) (Razavi, 2012) and *Ziziphus rotundifolia* seedlings (Arndt et al., 2001). In soybean (*Glycine max*), drought stress decreased leaf sucrose and starch concentrations but increased hexose (glucose and fructose) concentrations (Liu *et al.*, 2004). Soluble sugar concentration increased in roots and shoots of maize varieties when subjected to drought stress, but starch content significantly decreased in all evaluated varieties (Mohammadkhani and Heidari, 2008). The contents of soluble sugars, proteins, flavonoid and proline were higher in mycorrhizal than non-mycorrhizal plants under whole-water regime in pistachio seedlings (Abbaspour et al., 2011).

2.3.2.6 Root signaling under drought stress

Under drought stress conditions, roots induce a signal cascade to the shoots via xylem causing physiological changes eventually determining the level of stress adaptation. Root-shoot signals resulting from drought stress include abscisic acid (ABA), cytokinins, ethylene, malate and other unidentified factors (Anjum et al., 2011; Jaleel et al., 2009; Sacks *et al.*, 1997; Wu and Cosgrove, 2000). Panahi (2009) reported that ABA-levels in *Pistacia atlantica* subsp. *mutica* and *P. vera* Badami increased about 2-fold above the controls in drought-exposed plants, whereas in *P. vera* Sarakhs it showed a 1.5-fold increase.

2.3.3 Effect of drought stress on biochemical characteristics

2.3.3.1 Reactive Oxygen Species (ROS)

The production of ROS in plants is an early event of plant defense response to biotic and abiotic stress (Anjum et al., 2011; Hossain *et al.*, 2011; Mittler *et al.*, 2004), that generated ROS in chloroplasts, mitochondria and peroxisomes in the cells (Gill and Tuteja, 2010; Miller *et al.*, 2010).

Under progressive drought stress, electron transport will be impaired followed by an enhanced production of reactive oxygen species (Cornic and Fresneau, 2002; Cruz de Carvalho, 2008; Gnaana Saraswathi and Paliwal, 2011). Overproduction of ROS in plant cells under stress causes damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress (Gill and Tuteja, 2010; Rejeb *et al.*, 2014), and leads to destruction of photosynthetic pigments (Yordanov *et al.*, 2000). Proline protects cell membranes against the negative effects of ROS (Claussen, 2005; Matysik et al., 2002). Scavenging of ROS by enzymatic and non-enzymatic systems, cell membrane stability, expression of aquaporin and stress proteins are vital mechanisms of drought tolerance (Anjum et al., 2011; Farooq et al., 2009b; Ricardo, 2012).

2.3.3.2 Antioxidant defense system

Inadequate water supply under drought stress conditions promotes oxidative stress with overproduction of reactive oxygen species (ROS) (Apel and Hirt, 2004; Mittler et al., 2004). There is an internal defensive system (antioxidant defense system) in plants to avoid ROS injuries, thus guaranteeing normal cellular functioning (Apel and Hirt, 2004; Horváth *et al.*, 2007). Plants balance ROS through their antioxidant defense system with enzymatic and non-enzymatic components (Apel and Hirt, 2004; Hussain et al., 2008; Simova-Stoilova *et al.*,

2008). Non-enzymatic antioxidants cooperate to maintain the integrity of the photosynthetic membranes under oxidative stress. The enzymatic components may directly scavenge ROS or may act by producing a non-enzymatic antioxidant (Anjum et al., 2011; Apel and Hirt, 2004; Gill and Tuteja, 2010; Li, 2008; Scandalios, 2005).

Osmotic stress which is caused by water shortage conditions is often accompanied by compatible solutes accumulation and ionic toxicity. Osmotic stress under saline conditions, subjects plants to dehydration. Ionic toxicity results from the accumulation of specific ions, such as Na and Cl, in the cytoplasm or apoplast which interferes with plant metabolic functions. Under low to moderate salinity plants are adjusted osmotically by using part of their photosynthetic products to increase internal solute concentrations and thus do not show dehydration symptoms. Also, plants regulate their ionic balance to maintain normal metabolism (Pessarakli, 1994; Taiz and Zeiger, 2010).

Pistachio seedlings evaluation showed that arbuscular mycorrhiza colonization improved drought tolerance of *P. vera* seedlings by increasing the accumulation of osmotic adjustment compounds, and nutritional and antioxidant enzyme activity (Abbaspour et al., 2011). With the accumulation of solutes (soluble sugars, proteins, flavonoid, proline, phosphorous and potassium), the osmotic potential of the cell is lowered, which attracts water into the cell and helps with turgor maintenance. Osmotic adjustment helps to maintain the cell water balance with the active accumulation of solutes in the cytoplasm, thereby minimizing the harmful effects of drought (Maraghni *et al.*, 2014; Rouhi, 2007; Taiz and Zeiger, 2010).

Silicon treatment increased catalase (CAT), and superoxide dismutase (SOD) activities and decreased lipid peroxidation in leaves of drought-stressed pistachio plants (Habibi and Hajiboland, 2013). Antioxidant enzymes (CAT and SOD) protect plant structural and molecular components from the effects of ROS accumulated during drought stress (Apel and Hirt, 2004).

2.3.3.3 Nutrient relations

Nutrients elements such as nitrogen (N), phosphorous (P), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions play multiple essential roles in plant metabolism (Alam, 1999; Lahaye and Epstein, 1971). However, each of these nutrients must be maintained at an optimum concentration range for suitable growth (Ali et al., 2008; Ashraf *et al.*, 2013; Farooq et al., 2009b).

Decreasing water availability under drought generally results in limited total nutrient uptake and diminished tissue concentrations in crop plants (Farooq et al., 2009b). Normally,

decrease in nutrient uptake under water-deficit conditions takes place due to a reduction in transpiration rate (Ali et al., 2008; Jabeen *et al.*, 2008; Tanguilig *et al.*, 1987) and impaired active transport and membrane permeability resulting in reduced root absorbing power (Tanguilig et al., 1987).

Under drought stress conditions, available soil N (NO_3^- and NH_4^+) and N_2 fixation is greatly reduced, and such reduction leads to low N accumulation and consequently low dry matter production and low crop yield as was shown in peanut (*Arachis hypogaea* L.) (Pimratch *et al.*, 2008). Nutrient uptake in the latter plant was lower under drought stress conditions compared to control condition (Junjittakarn *et al.*, 2013).

Drought stress significantly decreased accumulation of essential nutrients (K^+, Ca^{2+}, N, and P) both in roots and shoots of canola (*Brassica napus* L.) cultivars (Ashraf et al., 2013) and maize (*Zea mays*). However, exogenous application of proline promoted the uptake of K^+, Ca^{2+}, N and P in maize (*Zea mays*) cultivars (Ali et al., 2008).

Drought stress decreased N content in plants, root and shoot dry weight of *Lactuca sativa* (Ruiz-Lozano and Azcón, 1996). Water stress caused a decrease in shoot biomass, nitrate reductase activity and an increase in nitrate content in the roots of *Vigna unguiculata* (da Silva *et al.*, 2011; Silveira *et al.*, 2001). Singh and Singh (2004) reported that calcium (Ca) contents increased around 200% and 270 % in roots and leaves, respectively, in *Dalbergia sissoo* seedlings under severe drought stress condition.

Fisher (1980) evaluated the responses of *Stylosanthes humilis* to water stress and found that phosphorus concentration was greatly reduced by water stress; P concentration in stems was more sensitive to stress than in leaves; and the P level returned to the same level as the control after the relief of stress.

Arbuscular mycorrhiza (AM) colonization increased pistachio P content under drought conditions. Results of Bagheri *et al.* (2012) also illustrated that AM symbiosis had a positive role in Zn and Mn uptake, while Fe and Cu were not affected in this study. In a glasshouse experiment, Ghasemi *et al.* (2015) reported that uptake of most of the mineral elements in pistachio leaves and roots was reduced by drought stress.

In a glasshouse experiment, Afrousheh *et al.* (2010) showed that height, leaf area and leaf number of pistachio seedlings decreased at various levels of nutrient element deficiencies. They also revealed that fractions of chlorophyll, transpiration, stomata conduction and stomatal resistance in treated seedlings, particularly N-deficient seedlings, significantly declined.

In summary, drought stress reduces the availability, uptake, translocation and metabolism of nutrients. A reduced transpiration rate due to water deficit reduces nutrient absorption and efficiency of their utilization (Farooq et al., 2009b).

2.3.3.4 Carbon isotope discrimination

Carbon isotope discrimination ($\Delta^{13}C$) is a measure of the $^{13}C/^{12}C$ ratio in plant material relative to the value of the same ratio in the air on which plants feed. The overall abundance of ^{13}C relative to ^{12}C in plant tissue is commonly less than in the carbon of atmospheric carbon dioxide, indicating that carbon isotope discrimination occurs in the incorporation of CO_2 into plant biomass (Condon *et al.*, 2004; Farquhar *et al.*, 1989). The carbon isotope composition ($\delta^{13}C$) is estimated by measurement of the $^{13}C/^{12}C$ ratio in a plant sample related to the value of the same ratio in an accepted international standard, the limestone Pee Dee Belemnite (PDB).

The dominant processes leading to carbon isotope discrimination are fractionations associated with CO_2 diffusion into leaf intracellular air spaces and with CO_2 carboxylation by the enzyme ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco= that catalysis CO_2 fixation in the Calvin cycle) and during the diffusion of CO_2 from the atmosphere to the chloroplast (Farquhar and Sharkey, 1982). Discrimination against ^{13}C in leaves during photosynthesis decreases with water stress, mainly because of the lowered stomatal conductance (Farquhar and Richards, 1984; Farquhar et al., 1989; Yousfi *et al.*, 2012b). Plants show a positive discrimination (Δ) against ^{13}C. Typically C_3 plants have a discrimination of~20×10^{-3}, which is normally presented in the literature as 20 ‰ (per mil). The discrimination factor of Rubisco is 29–30 per mil (‰), whereas discrimination during diffusion in air and the liquid phase are 4.4 and 1.8‰, respectively. The advantage of reporting Δ is that it directly expresses the consequences of biological processes, whereas composition, δp, is the result of both source isotopic composition and carbon isotope discrimination (Farquhar et al., 1989). Some authors predicted a strong linear relationship between carbon isotope discrimination (Δ) and the ratio of intercellular to ambient CO_2 partial pressure (C_i/C_a). However, internal diffusion of the CO_2 from the substomatal cavity to site of Rubisco carboxylation in the chloroplast stroma also affects discrimination and results in a dependency of Δ on photosynthetic rates. For example, Δ can vary by 6–8 ‰ with variation in rates of photosynthesis driven by changes in light intensity (Evans, 1989; Farquhar et al., 1989).

Carbon isotope discrimination resolution ($\Delta^{13}C$) and carbon isotope composition ($\delta^{13}C$) are two commonly used indices in the stable carbon isotope discrimination. $\Delta^{13}C$ can reflect the change of *Ci*/Ca [the ratio of leaf intercellular CO_2 concentration (*Ci*) to the ambient atmospheric CO_2 concentration (Ca)] in a time interval and it can indirectly express the deviation of the leaf intercellular $^{13}C/^{12}C$ related to the ambient atmosphere $^{13}C/^{12}C$ on the basis of plant dry mass. Such carbon isotope discrimination can directly reflect the impendent difference in carboxylation reaction of ribulose diphosphate carboxylase (RUBPC) and phosphoenolpyruvate carboxylase (PEPC) as well as the diffusion of CO_2 in the interior of leaf (Dawson *et al.*, 2002; Farquhar and Sharkey, 1982).

Negative correlations between $\Delta^{13}C$ and water use efficiency have been demonstrated in many species (Farquhar et al., 1989). Carbon isotope discrimination has become a tool to help us understand photosynthesis and its coordination with water use in ecological and physiological studies of C_3 species (Del Amor and Cuadra-Crespo, 2011; Farquhar et al., 1989). These observations point to $\Delta^{13}C$ as a potential candidate for use in breeding for greater agronomic water use efficiency (Condon et al., 2004).

The $\delta^{13}C$ of plant samples which is analyzed by an isotope mass-spectrometer can integrate the carbon absorption of all organs and the change of photosynthetic characteristics and physiological indices in the plant growth, so it can be used as a suitable parameter to reveal the water stress effects. Thus it can evaluate the accuracy of mean leaf WUE determined by the gas exchange (Cui *et al.*, 2009; Su *et al.*, 2004). Carbon isotope composition ($\delta^{13}C$) is measured by use of a mass spectrometer, which yields the following ratio:

$R= {}^{13}CO_2/{}^{12}CO_2$

The isotope composition of plants, $\delta^{13}C$, is quantified on a per mil (‰) basis:

$\delta^{13}C\ ‰= (R_{sample}/R_{standard}-1) \times 1000$

Where the standard represents the carbon isotopes contained in a fossil belemnite from the Pee Dee limestone formation of South Carolina. The $\delta^{13}C$ of atmospheric CO_2 has a value of –8 ‰, meaning that there is less ^{13}C in the atmospheric CO_2 than the one which is found in the carbonate of the belemnite standard (Farquhar and Richards, 1984). C_3 plants have a $\delta^{13}C$ of about –28 ‰; C_4 plants have an average value of –14 ‰ (Farquhar et al. 1989). Both C_3 and C_4 plants have less ^{13}C than the isotope standard, which means that there has been a discrimination against ^{13}C during the photosynthetic process. Plants also fractionate other isotopes, such as $^{15}N/^{14}N$ and $^{18}O/^{16}O$, and the various patterns of isotope enrichment or

depletion can be used as indicators of particular metabolic pathways or features (Cernusak *et al.*, 2008).

The analysis of the natural abundances of stable carbon isotopes in plant dry matter ($\delta^{13}C$) provides information on the long-term water use efficiency of plants, and values of $\delta^{13}C$ are determined mainly by three processes: diffusion of CO_2 through stomata, CO_2 assimilation by carboxylase, and metabolism of compounds (Farquhar et al., 1989).

The carbon isotope discrimination ($\Delta^{13}C$) of shoots is calculated as:

$\Delta^{13}C$ (‰) = $(\delta^{13}C_a - \delta^{13}C_p)/ [1+ (\delta^{13}C_p) /1000]$

Where the subscripts a and p refer to air and the plant, respectively (Farquhar et al., 1989).

Root and shoot carbon isotope composition ($\delta^{13}C$) levels increased in drought stress treatments compared with control in durum wheat (*Triticum turgidum* L.). Livingston *et al.* (1999) demonstrated there was a positive relation between $\delta^{13}C$, WUE and dry matter production, so that it should be possible to use $\delta^{13}C$ as a surrogate for WUE, and to select for increased WUE without compromising yield, even in nitrogen deficient environments in irrigated and dry land white spruce (*Picea glauca* (Moench) Voss) seedlings. Results of a greenhouse experiment on *Populus deltoids* clones under water stress conditions showed that there was a good positive correlation between $\delta^{13}C$ and leaf WUE (WUE_L) in the same water treatment, and that a high WUE_L always coincided with a high $\delta^{13}C$. Therefore, the authors concluded that carbon isotope composition ($\delta^{13}C$) might be a reliable indirect index to estimate WUE_L among *P. deltoids* clones (Zhao *et al.*, 2006a).

Carbon isotope discrimination (Δ) decreased with increasing salinity in leaves, stems and roots of pistachio (*P. vera*) seedlings. However, there was no significant difference in carbon isotope discrimination between three *P. vera* (Sarakhs, Badami-Zarand, and Ghazvini) rootstocks under the same treatments (Hokmabadi et al., 2005). Carbon isotope ratios of *Pistacia lentiscus, Quercus ilex* and *Phillyrea argustifoli* showed similar $\delta^{13}C$ values, while two deciduous oak species, had lower $\delta^{13}C$ values in a Mediterranean ecosystem (Valentini *et al.*, 1992), therefore these results show that the carbon isotope discrimination values can vary in different plant species.

2.3.3.5 Nitrogen isotope discrimination

Nitrogen exists as two naturally occurring stable isotopes, ^{15}N and ^{14}N. Variation in the absolute abundance of ^{15}N is small, therefore, nitrogen isotope composition is expressed using δ notation in parts per thousand (per mil):

$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ ‰

Where $\delta^{15}N$ is the isotope ratio related to the atmospheric air standard, and R sample and R standard are the molar ratios of the heavier to the lighter isotope. Atmospheric nitrogen is the internationally recognized standard with an R value of $(^{15}N/^{14}N)_{std}$ of 0.0036765 (Poupin *et al.*, 2014). Differences in $\delta^{15}N$ between a substrate and product will occur when ^{15}N and ^{14}N react at different rates.

The nitrogen isotope discrimination ($\Delta^{15}N$) is calculated as:

$\Delta^{15}N$ (‰) = $(\delta^{15}N_a - \delta^{15}N_p)/ (1+ (\delta^{15}N_p) /1000)$

Where the subscripts a and p refer to air (standard) and the plant (sample), respectively (Farquhar et al., 1989).

The natural variation in plant N isotope composition ($\delta^{15}N$) is potentially useful for genotypic screening under drought or salinity because it is linked to N metabolism, even though a complete knowledge of the underlying biochemical mechanisms is lacking (Cernusak et al., 2008). Isotope fractionation may occur during enzymatic assimilation of nitrate or ammonium into other N forms.

Discrimination is positive in most biological systems; therefore, the product should have a lower $\delta^{15}N$ value than the substrate (Farquhar 1989). Water availability is the primary environmental factor controlling the variability of plant $\delta^{13}C$ and $\delta^{15}N$ and soil $\delta^{13}C$ in the arid and semi-arid regions (Ma *et al.*, 2012). The stable nitrogen isotope ratio ($\delta^{15}N$) in the fruit mesocarp of peach trees (*Prunus persica*) was significantly related to the amount of N applied and the origin of this N. The nitrogen isotope ratio did not, however, exhibit a good discriminative capacity when they evaluated the influence of water and nitrogen on plant response (Pascual *et al.*, 2013).

Root $\delta^{15}N$ increased in all stress treatments in dry matter of durum wheat (*Triticum aestivum*), showing the highest values in the two most stressful treatments. By contrast, stress treatments significantly decreased shoot $\delta^{15}N$ (Yousfi et al., 2012b).

Moreover, root nitrogen isotope composition ($\delta^{15}N$) increased in stressed treatments, although stress treatments significantly decreased shoot $\delta^{15}N$ in durum wheat (Yousfi *et al.*, 2012a). High salt concentrations significantly decreased nitrogen isotope discrimination in leaf dry matter of broccoli (*Brassica oleracea* L.) (Del Amor and Cuadra-Crespo, 2011).

Chapter 3. **Effects of osmotic drought stress on physiological responses, growth characteristics, mineral elements and carbon isotope composition of three pistachio (*Pistacia* spp.) rootstocks**

Adapted from:

Esmaeilpour, A.**,** M.C. Van Labeke, R. Samson, P. Boeckx and P. Van Damme. 2015. Impact of osmotic drought stress on carbon isotope discrimination and growth parameters in three pistachio rootstocks. XVI GREMPA Meeting on Almonds and Pistachios. May 12-14, Meknes, Morocco.

Esmaeilpour, A., M. C. Van Labeke, R. Samson, P. Boeckx and P. Van Damme. 2017. Effects of osmotic drought stress on physiological responses, growth characteristics, mineral elements and carbon isotope composition of three pistachio rootstocks (submitted to journal).

3.1 Introduction

The amount of water available for plant growth is mainly influenced by water resource limitation which depends on climate and soil conditions. Any water content of plant tissues or cells that is below the highest water content at the fully hydrated state is defined as water deficit or osmotic stress (Taiz and Zeiger, 2010). Drought stress adversely affects growth, dry mass accumulation, and productivity of plants (Anjum et al., 2011; Zhao *et al.*, 2006b) whereas it also causes a higher rate of impairment than any other environmental stress factor (Shao et al., 2009). Still, severe water deficit can cause a reduction in plant growth, and eventually yield and nut quality in pistachio plants. These harsh growing conditions already led to the loss of important local genetic resources of pistachio cultivars and rootstocks (Panahi et al., 2002).

Wild pistachio species are very often used as rootstock for *P. vera* plants. Germana (1996) found that midday xylem sap water potentials of plants grafted on *P. atlantica* or *P. terebinthus* did not drop below -0.5 MPa under drought conditions, and explained this by the latter species' deep, extensive root systems. It is in this context that drought stress effects were evaluated for *P. vera* cv. Kerman grafted onto three different pistachio rootstocks. Grafting onto UCB#1 pistachio rootstock (hybrid from *P. atlantica* * *P. integerrima*) and on *P. terebinthus* resulted in a higher growth reduction compared to plants grafted on *P. atlantica* under drought stress (Gijón *et al.*, 2010). However, physiological adaptations of pistachio to drought stress have only been studied to a limited extent. Comparing two pistachio rootstocks with non-drought stressed plants, Bagheri et al. (2011) found that Qazvini cultivar was more tolerant to drought stress than Badami cultivar as the first maintained a higher photosynthetic activity under drought. Net photosynthetic rates were more reduced for *P. mutica* than for *P. khinjuk* under increasing osmotic drought stress, indicating a higher drought tolerance for *P. khinjuk* (Ranjbarfordoei et al., 2000).

Carbon isotope discrimination (Δ) decreases with increasing salinity in leaves, stems and roots of *P. vera* seedlings. However, no significant difference in carbon isotope discrimination between three *P. vera* (Sarakhs, Badami-Zarand, and Qazvini) rootstocks was evidenced by Hokmabadi et al. (2005). Carbon isotope ratios of *P. lentiscus*, *Quercus ilex* and *Phillyrea argustifoli* showed equivalent $\delta^{13}C$ values, while two deciduous oak species, *Q. pubescens* and *Q. cerris*, had lower $\delta^{13}C$ values in a Mediterranean ecosystem (Valentini et al., 1992) .

Root and shoot $\delta^{13}C$ values increased in stress treatments compared with those of full-irrigation plants in durum wheat (*Triticum turgidum* L.), but in the latter case, the highest values were observed under the most stressful conditions (Yousfi et al., 2012a).

Stable carbon isotope ratio ($\delta^{13}C$) in fruit of peach trees (*Prunus persica* L.) proved a reliable indicator of plant water status and physiological water use efficiency (WUE). Leaf carbon isotope ratio was not affected by either irrigation or nitrogen (N) treatments (Pascual et al., 2013). Salinity significantly increased carbon isotope discrimination in dry leaf matter of broccoli (*Brassica oleracea* L.) (Del Amor and Cuadra-Crespo, 2011).

There are three pistachio species (*P. vera*, *P. khinjuk* and *P. atlantica* subspecies *mutica*) in Iran (Esmaeilpour and Khezri, 2006) which are growing under different environmental conditions (altitude 900-2000 m; latitude 24-37 N; temperatures ranging between −20 °C in winter and 42 °C in summer, low to moderate humidity, and long, hot summers). Response to osmotic drought stress of two of these three species (*P. khinjuk* and *P. atlantica* subspecies *mutica*) have been investigated by Fardooei (2001). *Pistacia vera* L. is the country's most common rootstock. Yet, physiological responses of this rootstock to drought stress and comparison with other recommended rootstocks have not been studied extensively.

The aim of the present study was to evaluate the effects of osmotic drought stress treatment and subsequent recovery period on physiological performance of *P. vera* cv. Badami, *P. vera* cv. Sarakhs (native), and *P. terebinthus* rootstocks (used in Turkey). Plant responses were assessed via chlorophyll fluorescence, mineral element content, carbon isotope composition and a number of growth characteristics. We hypothesized that chlorophyll fluorescence, and carbon isotope composition could be used as indices for the selection of drought-tolerant pistachio rootstocks.

3.2 Materials and methods

3.2.1 Plant material and experimental set-up

This study was carried out in a glasshouse of the Faculty of Bioscience Engineering, Ghent University (51°3' N, 3°42' E). Certified seeds of *Pistacia vera* L. cv. Badami (BA) and *P. vera* L. cv. Sarakhs (SA) (Figure 3.1, A and B) were obtained from the Iranian Pistachio Research Institute (IPRI), Rafsanjan, Iran. *P. terebinthus* (TER) seeds were obtained from the pistachio production areas in Gaziantep, Turkey (Figure 3.1, C). Badami and Sarakhs seeds were first soaked in water for 12 hours and then pre-treated for 20 minutes with 0.01% captan (N-trichloromethyl-thio-tetrahydro phthalimide) solution, a broad-spectrum fungicide (Panahi

et al., 2002). Seeds of *P. terebinthus* were scarified with sulfuric acid for 10 min and subsequently washed three times in distilled water. In June 2010, seeds were sown in 4-L pots containing sand and organic material. Planting were managed according to good agricultural management as described by Panahi et al. (2002) during the first growing season. In March 2011, seedlings were transplanted to 5-L pots filled with vermiculite. Transplanted 1-year-old seedlings were grown hydroponically in a controlled glasshouse environment using standard Hoagland (See Appendix 3- chapter 3) solution (Picchioni *et al.*, 1991) for fertigation (Figure 3.2). Averages of minimum and maximum temperatures in the glasshouse were 15 ºC and 36 ºC, respectively.

Figure 3.1: Seeds of different pistachio rootstocks (A- *Pistacia vera* cv. Badami; B- *P. vera* cv. Sarakhs and C- *P. terebinthus*) to study the response of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

In May 2011, osmotic drought treatments were applied. They consisted of a control (osmotic potential of the nutrient solution (Ψ_s) = -0.10 MPa) and three drought stress levels (mild stress, $\Psi_{s=}$ -0. 5 MPa, moderate stress $\Psi_{s=}$ - 1.0 MPa and severe stress, $\Psi_{s=}$ -1.5 MPa) using PEG 6000 (Ranjbarfordoei et al., 2002). The concentration of PEG 6000 (g kg $^{-1}$ water) was determined for each drought stress treatment according to the Michel BE and Kaufmann (1973) equation which is as follows:

(3.1)

$$\Psi s = -(1.18 \times 0.01) \times C - (1.18 \times 0.0001) \times C \times C + (2.67 \times 0.0001) \times C \times T + (8.39 \times 0.0000001) \times C \times C \times T$$

Where:

Ψ_s is osmotic potential of aqueous solution in bar, C is concentration of PEG in g/kg water, T is temperature of the aqueous solution, in˚C (here 23 to 25˚C). Final water potential for each treatment was calculated using the following equation according to Michel BE and Kaufmann (1973).

(3.2)

$$\Psi s = \Psi PEG + \Psi f + \Psi sy$$

(3.3)

$$\Psi sy = 0.07 \times (\Psi s + \Psi f)$$

Where:

Ψ_s is osmotic potential of irrigation water, Ψ_{PEG} is osmotic potential of PEG 6000 in pure water, Ψ_f is osmotic potential of fertilizer (Here, Hoagland solution), Ψ_{sy} is synergy effect potential between PEG 6000 and fertilizer. The osmotic potential of Hoagland solution in the tap water was -0.08 MPa. For example, water potential of 180 g PEG in one kg water at 25℃ with Hoagland solution is:

Ψ_{PEG}= -0.41 MPa potential of PEG 6000 in pure water, Ψ_f = -0.08 MPa potential produced by fertilizer, Ψ_{sy} = 0.07 × [(-0.41) + (-0.08)] = -0.343 MPa potential of synergistic effect, Ψ_s = (-0.41) + (-0.08) + (-0.0343) = -0.52 MPa final water potential.

Stress levels were maintained for two weeks; then all solutions were replaced by the control treatment, and this level was maintained for two recovery weeks.

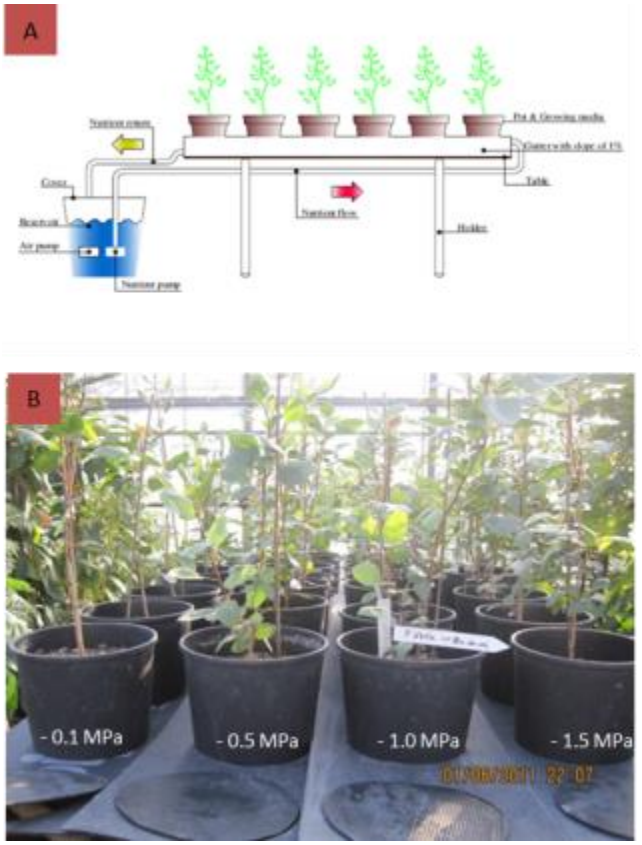


Figure 3.2: Experimental set up to study the response of pistachio rootstock seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

3.2.2 Measurements

3.2.2.1 Chlorophyll fluorescence measurements

Measurements were taken on one fully expanded leaf using a portable chlorophyll fluorometer (PAM-2500, Heinz Walz GmbH, Effeltrich, Germany) (Figure 3.3, A and B) in five replicates (plants) per cultivar (Fardooei, 2001; Rouhi, 2007). Chlorophyll fluorescence parameters were measured after 14 days of drought stress and after 14 days of recovery between 9 am and 3 pm. To allow dark adaptation of the leaves, before measuring, 10 dark leaf clips were put on 10 plants (replicates) at the same time and kept for 30 minutes, after which leaves were measured one by one. Every single measurement took about 4-5 minutes. After 30 min of dark-adaptation, F_v/F_m was calculated as $(F_m - F_0)/F_m$, where F_m [induced by a short pulse (0.6 s) of saturating light (3,450 µmol m^{-2} s^{-1})] and F_0 were the maximal and minimal fluorescence, respectively (Genty et al., 1989). After 4 min of illumination with continuous red, non-saturating actinic light (447 µmol m^{-2} s^{-1}) and with saturating pulses

(3,450 µmol m^{-2} s^{-1}) every 25 s, maximum (F'_m) and steady state (F_s) fluorescence signals were measured in light-adapted leaves. Then, the actinic light was turned off, and a far red pulse was applied to obtain minimal fluorescence after PSI excitation (F_0'). YII was calculated as ($F'_m - F_s$)/ F'_m and qP was calculated as ($F'_m - F_s$)/ ($F'_m - F'_0$) (Kooten and Snel, 1990). NPQ, which is proportional to the rate constant for thermal energy dissipation, was estimated as ($F^0_m - F'_m$)/ F'_m . The electron transport rate (ETR) was calculated as YII x PAR x 0.84 x 0.5, where the absorbed photon energy (PAR) is assumed to be equally distributed between PSI and PSII, and 0.84 is the assumed light absorptance of the leaf.

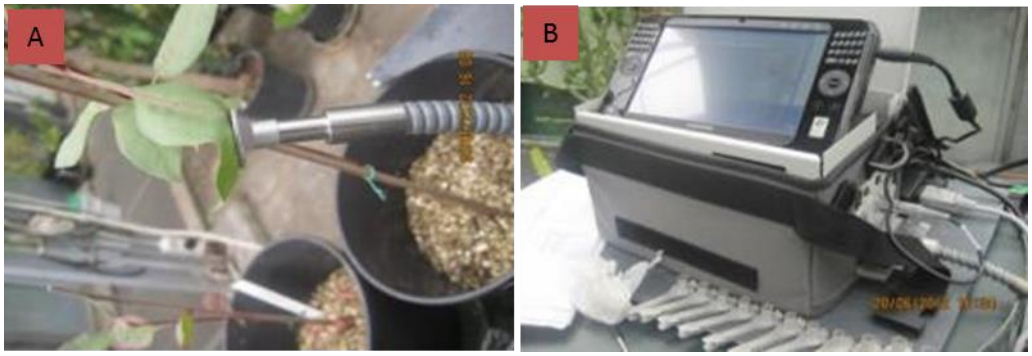


Figure 3.3: Measurement of leaf chlorophyll fluorometer parameters (A and B) of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

3.2.2.2 Leaf mineral element content

At each treatment, all leaves were sampled from every plant for each rootstock, and these were combined to form a single sample for later analyses. Plants were sampled once after harvesting the whole plants. Leaf tissues were oven dried at 85 °C for 72 hours (Jouan laboratory oven, UK) and then ground to a 40 mesh in a Wiley mill. Dried leaf samples were used to determine the mineral element concentration. Each sample was replicated five times for every treatment and rootstock. Phosphorous (P), calcium (Ca), magnesium (Mg), iron (Fe) and zinc (Zn) were determined by atomic absorption spectrophotometry using respective hollow cathode tubes (Ehleringer *et al.*, 1986) whereas potassium (K) was estimated in a known volume of acid extract using a flame photometer (Hald, 1947).

3.2.2.3 Carbon isotope composition

Dried leaf samples were ground with a grinder (ZM200, Retsch, Germany) (Figure 3.4) in the Isotope Bioscience Laboratory (ISOFYS), Department of Applied Analytical and Physical Chemistry, Faculty of Bioscience Engineering, Ghent University. Five mg subsamples of ground plant material (three replicates per treatment and rootstock) were packed in tin capsules and analyzed for natural abundance of ^{13}C by combustion to CO_2 in the present of O_2 by an elemental analyzer (EA) (ANCA-SL, SerCon, UK) coupled to an isotope ratio mass spectrometer (IRMS) (model 20–20, SerCon, UK) to measure $\delta^{13}C$ (Staelens *et al.*, 2012). Carbon contents were expressed as a percentage of dry matter. Carbon isotope composition (δ) was calculated relative to the international Pee Dee Belemnite (PDB) standard. The following formula was used to calculate carbon isotope composition: $\delta^{13}C = [(R_{sa}/R_{sd} -1) \times 1000]$ (‰) where R_{sa} and R_{sd} are the ^{13}C:^{12}C ratios of the sample and standard, respectively (Farquhar et al., 1989). The $\delta^{13}C$ value of the air was taken as - 0.008.

Figure 3.4: Grinding of leaf pistachio rootstock seedlings to measure carbon and nitrogen isotope contents exposed to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

3.2.2.4 Plant growth parameters

At the end of the second recovery week, all seedlings were harvested. Fresh weight of leaves, shoots and roots was determined with an electronic precision balance of ± 0.1 mg (Mettler Toledo PB602-L, Greifensee, Switzerland), in 5 replicates per treatment (Figure 3.5, A-E). The dry weight of the respective plant fractions was determined after drying at 85 °C for 72 hours (Jouan laboratory oven, UK). Root/shoot ratio was calculated by dividing total below-ground to total aboveground biomass (R/S ratio). Leaf area per plant was determined by means of a planimeter. Fallen leaves were collected during the experiment and weighted

in every treatment to assess leaf shedding. Plant height of each plant was measured from the base (the point where roots separate from the stem) at the beginning (H_1) and end of the experiment (H_2) with a ruler (± 0.1 cm). The stem elongation (SEL) was calculated using the following equation:

$$SEL = H_2 - H_1 \qquad (5.1)$$

Where, H_1 is stem height before imposition of drought stress and H_2 is stem height at the end of the experiment.

Figure 3.5: Measurement of fresh and dry weights of different parts (A-E) of pistachio rootstock seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

3.2.3 Statistical analysis

The experiment was planned as a randomized complete design (RCD). Each experimental unit contained 5 plants whereas a total of 20 plants were used for the four treatments in every rootstock. A two-way analysis of variance was used to test for effects of drought treatments * rootstocks. One-way ANOVA to test treatments by rootstock was also performed. Means were compared using Tukey's HSD test ($P<0.05$). Correlations between parameters were calculated using Pearson's correlation method. Principal component analysis (PCA) was carried out on all measured parameters. Only principal components with eigenvalues > 1, thus explaining more than a single parameter alone, were extracted. In this experiment, we have 3 rootstocks × 4 treatments/water stresses (3 × 4= 12 treatment levels) with 5

replications and 21 variables/vectors. Therefore, we applied PCA to a 60 × 21 matrix. In the biplot the mean values of the replications were inserted, while all data were used for PCA and all other statistical analyses. Multivariate analysis of variance (MANOVA) was conducted for all cultivars and measured parameters. A normality test of data distribution was conducted using descriptive statistical parameters and a homogeneity test was done to see diverse data using Levene's test of equality of error variance by SPSS software. All analyses were performed in SPSS 20 (IBM Corporation, USA).

3.3 Results

3.3.1 Multivariate analysis of variance (MANOVA)

The MANOVA analysis (Table 3.1) revealed significant differences among three rootstocks indicating the presence of genotypic variability. There are significant differences among four drought stress conditions (treatments) by considering all parameters. Interaction effect of rootstock * treatment was significant, indicating different responses of genotypes to water stress condition and possible selection of drought tolerant genotypes under water deficit.

Table 3.1: Multivariate analysis of variance (MANOVA) for all measured parameters under different environmental conditions in three pistachio rootstocks.

Effect	Test	Value	F	Hypothesis df	Error df	Sig.
Rootstock	Roy's Largest Root	561.22	51.02	22	2	0.019
Treatment	Roy's Largest Root	134.41	18.33	22	3	0.017
Rootstock* Treatment	Roy's Largest Root	651.90	177.79	22	6	<0.001
Block	Roy's Largest Root	217.02	19.73	22	2	0.05

3.3.2 Chlorophyll fluorescence

Increasing osmotic drought stress significantly decreased quantum yield of photosystem II (YII) and photochemical quenching (qP) during drought stage in all three rootstocks, but differences were not significant between drought stress treatments (Table 3.2). No significant

differences in maximum quantum yield of PSII ($F_v/F_{m,}$) and non-photochemical quenching (NPQ) under increasing stress were observed for the three rootstocks compared with control plants (Table 3.2). At drought stage, values for YII and qP in Badami were higher ($P< 0.01$) than Sarakhs and *P. terebinthus* rootstocks, while NPQ was higher in *P. terebinthus* compared to the other two rootstocks (Table 3.2).

During recovery, F_v/F_m ratios and YII remained lower compared to the parameters levels in control plants in Terebinthus and Sarakhs, although there were no significant differences between the applied drought stress treatments. Values of qP in exposed plants also were lower than corresponding control plants in *P. terebinthus* rootstock (Table 3.2). Overall, the effects of drought stress treatments on the above-measured parameters (F_v/F_m ratios, YII and qP) were significantly lower after two weeks of recovery, as values of YII and qP were the highest in Badami, NPQ was highest in *P. terebinthus* and F_v/F_m was highest in Sarakhs in recovery stage (data not shown).

ANOVA analysis showed that rootstock effects on all chlorophyll fluorescence parameters were significant during both drought and recovery stages (except F_v/F_m in drought). Effects of treatments on YII, qP ($P< 0.01$) in drought stage and on F_v/F_m, YII ($P< 0.01$) and qP ($P< 0.05$) were significant in recovery stage. Rootstocks x treatments interaction were not significant for any parameters (except NPQ) during drought and recovery stages (Table 3.2).

3.3.3 Plant growth parameters

Drought stress significantly decreased plant fresh weight (PFW), plant dry weight (PDW), shoot dry weight (SDW) and leaf dry weight (LDW) compared to the control plants in *P. terebinthus* rootstock, though differences between drought stress treatments were not significant (Table 3.3). There were no significant differences between drought stress treatments and the control treatment for these parameters in Badami and Sarakhs rootstocks. Although root dry weights were not affected by osmotic stress treatments, root/shoot ratio increased in all three rootstocks and it was significant in Badami rootstock (Table 3.3).

Plant's leaf area was significantly decreased by drought stress treatments in Badami and *P. terebinthus*, though there was no significant difference between control and drought-exposed plants in Sarakhs. Control plants showed stem elongation during the experimental period, but significant growth arrest were noted under drought stress treatments in all three rootstocks (Table 3.3). Decreasing osmotic potential of the nutrient solution increased leaf shedding contents, although differences were not significant (Table 3.3). Effects of rootstocks were

significant for all growth parameters, while treatment effects were significant for PFW, PDW, SDW, LDW, R/S ratio, leaf area and stem elongation. ANOVA analysis showed that interaction effects were not significant for any parameter, except for leaf area and stem elongation (Table 3.3). Terebinthus rootstock showed the highest values for the considered biomass parameters, leaf area and stem elongation, whereas Badami had the most pronounced leaf shedding compared to the other rootstocks (data not shown).

Table 3.2: Changes in chlorophyll fluorescence parameters (F_v/F_m, YII, NPQ, qP) in three pistachio rootstocks (R) during a period of osmotic drought stress (T, MPa) and subsequent recovery. Values are means ± SE of five replicates.

		DROUGHT				**RECOVERY**			
		F_v/F_m	YII	NPQ	qP	F_v/F_m	YII	NPQ	qP
Badami	-0.1	0.72±0.02 a	0.30±0.01 a	0.83±0.12 a	0.60±0.02 a	0.78±0.01 a	0.24±0.04 a	1.20±0.07 a	0.49±0.08a
	-0.5	0.75±0.01 a	0.18±0.01 b	1.20±0.1 a	0.38±0.01 b	0.78±0.01 a	0.22±0.02 a	1.47±0.12 a	0.49±0.05 a
	-1.0	0.72±0.03 a	0.15±0.01 b	1.08±0.05 a	0.34±0.02 b	0.78±0.01 a	0.19±0.01 a	1.41±0.16 a	0.40±0.05 a
	-1.5	0.63±0.07 a	0.14±0.02 b	0.83±0.17 a	0.35±0.03 b	0.77±0.01 a	0.24±0.01 a	1.04±0.17 a	0.49±0.02 a
Terebinthus	-0.1	0.73±0.01 a	0.22±0.00 a	1.18±0.07 a	0.54±0.02 a	0.79±0.01 a	0.19±0.01 a	1.59±0.18 a	0.45±0.04 a
	-0.5	0.71±0.02 a	0.11±0.02 b	1.45±0.14 a	0.30±0.03 b	0.77±0.01 ab	0.11±0.01 b	1.63±0.11 a	0.28±0.03 b
	-1.0	0.72±0.03 a	0.14±0.02 b	1.17±0.12 a	0.32±0.05 b	0.77±0.01 b	0.14±0.01 b	1.68±0.10 a	0.35±0.03 ab
	-1.5	0.68±0.04 a	0.10±0.01 b	1.23±0.21 a	0.27±0.01 b	0.77±0.01 ab	0.13±0.00 b	2.05±0.06 a	0.42±0.03 a
Sarakhs	-0.1	0.77±0.01 a	0.24±0.03 a	1.03±0.14 a	0.47±0.04 a	0.81±0.00 a	0.26±0.01 a	1.22±0.07 a	0.48±0.02 a
	-0.5	0.73±0.02 a	0.14±0.01 b	0.81±0.06 a	0.28±0.01 b	0.79±0.01 ab	0.19±0.02 b	1.06±0.07 a	0.35±0.04 a
	-1.0	0.73±0.02 a	0.14±0.01 b	0.93±0.07 a	0.29±0.03 b	0.78±0.01 b	0.20±0.02 ab	1.44±0.12 a	0.43±0.05 a
	-1.5	0.73±0.03 a	0.13±0.01 b	0.71±0.11 a	0.26±0.02 b	0.78±0.00 b	0.18±0.02 b	1.24±0.19 a	0.38±0.05 a
ANOVA	R	ns	**	**	**	*	**	**	*
	T	ns	**	ns	**	**	**	ns	*
	R × T	ns	ns	ns	ns	ns	ns	*	ns

Within each column and for each rootstock, means superscripted with different letters are significantly different (** - (P< 0.0 1), * - (P <0.05) and ns- non significant).

Table 3.3: Effects of osmotic stress treatments (T, MPa) on plant fresh weight (PFW, g), plant dry weight (PDW, g), shoot dry weight (SDW, g), leaf dry weight (LDW, g), root dry weight (RDW, g), root/shoot dry weight (R/S ratio), leaf area (LA, cm^2), stem elongation (SEL, cm) and shedding (Shed, g) after two weeks of recovery for three pistachio rootstocks (R). Values are means ± SE of five replicates.

		PFW	PDW	SDW	LDW	RDW	R/s Ratio	L A	SEL	Shed
Badami	-0.1	50.48±26.64 a	17.18±9.22 a	11.34±6.44 a	5.24±3.48 a	5.82±3.31 a	0.54±0.18 b	15.02±4.46 a	19.7±10.84 a	0.0±0 a
	-0.5	25.48±6.62 a	10.84±3.3 a	5.74±1.5 a	2.0±0.47 ab	5.1±2.05 a	0.88±0.25 a	12.6±3.92 b	1.1±0.55 b	0.46±0.45 a
	-1	29.74±11.98 a	10.62±4.44 a	5.72±2.3 a	1.88±1.19 ab	4.88±2.16 a	0.85±0.09 ab	11.36±3.31 b	0.94±0.8 b	0.32±0.25 a
	-1.5	22.96±7.06 a	9.9±2.51 a	5.52±1.5 a	1.82±0.54 b	4.38±1.26 a	0.80±0.2 a	9.52±2.64 c	1.1±0.82 b	0.31±0.21 a
Terebinthus	-0.1	75.18±31.18 a	24.24±9.54 a	16.4±7.51a	10±5.09 a	7.84±2.79 a	0.64±0.5 a	42.26±15.37 a	24±13.93 a	0.06±0.08 a
	-0.5	43.16±9.11ab	17.22±4.07 ab	9.88±2.81ab	5.64±1.67 ab	7.36±1.98 a	0.77±0.23 a	28.06±6.5 b	1.82±0.74 b	0.0±0 a
	-1	30.12±13.42 b	12.22±5.97 b	6.84±3.48 b	3.82±2.2 b	5.4±2.58 a	0.83±0.19 a	27.02±5.62 b	0.94±0.77 b	0.13±0.17 a
	-1.5	31.46±7.7 b	12.82±3.06 b	6.86±1.59 b	3.96±1.61 b	6.0±1.66 a	0.88±0.16 a	28.12±4.85 b	4.14±4.86 b	0.06±0.11 a
Sarakhs	-0.1	32.9±13.74 a	13.3±5.12 a	7.26±2.44 a	3.02±1.31a	6.04±2.75 a	0.82±0.14 a	10.98±4.9 a	4.96±8.7 a	0.0±0 a
	-0.5	20.52±10.38 a	9.58±4.67 a	5.04±2.61a	1.9±1.21a	4.54±2.13 a	0.91±0.13 a	15.54±6.85 a	1.8±1.6 b	0.04±0.01 a
	-1	17.86±8.9 a	8.4±4.3 a	4.18±2.22 a	1.82±1.14 a	4.22±2.29 a	1.01±0.26 a	12.88±8.71 a	0.96±1.26 b	0.08±0.01a
	-1.5	18.98±7 a	8.86±3.34 a	4.24±1.52 a	1.68±0.86 a	4.64±2.23 a	1.11±0.47 a	12.38±2.08 a	1.56±1.74 b	0.09±0.01 a
ANOVA	R	**	**	**	**	*	*	**	*	**
	T	**	**	**	**	ns	*	*	**	ns
	R× T	ns	ns	ns	ns	ns	ns	*	**	ns

Within each column and for each rootstock, means superscripted with different letters are significantly different (** - (P< 0.0 1), * - (P <0.05) and ns- non significant).

3.3.4 Leaf mineral elements

A decreasing osmotic potential of the nutrient solution significantly decreased leaf phosphorus (P) concentration compared to that of control plants in Badami and Sarakhs rootstocks, while differences were not significant in Terebinthus (Table 3.4). Drought stress treatments significantly decreased leaf concentration of potassium (K) in Badami but not in other two rootstocks. Calcium (Ca) contents decreased in Badami (significant) and Sarakhs rootstocks, though it significantly increased in Terebinthus rootstock. Magnesium (Mg) concentration significantly decreased under drought stress treatments in Badami. Leaf iron (Fe) concentrations were not affected by drought stress treatments in either three rootstocks. Concentration of leaf zinc (Zn) increased in severe drought stress treatment compared to control in all three rootstocks, though it was significant in Terebinthus rootstock (Table 3.4).

ANOVA analysis showed that effects of rootstock on all leaf mineral elements except Fe were significant when they were exposed to drought stress conditions. While treatment effects were none significant on all leaf mineral elements except P. The interaction effects were only significant for leaf P and Ca concentrations (Table 3.4). There were significant negative correlations between Fe and Zn with PDW (Table 3.5). The correlation between Mg and PDW was significant (Table 3.5).

3.3.5 Carbon isotope composition

Increasing drought stress intensity increased the values of leaf carbon isotope composition of all three rootstocks, although differences were not significant. ANOVA analysis showed that the effects of rootstock on carbon isotope composition were significant (Table 3.4). Leaf carbon isotope composition was highest in Sarakhs (-28.47‰), average in Badami (-29.54‰) and lowest in Terebinthus (-30.31‰) rootstocks (data not shown). There were significant positive correlations between carbon isotope composition with F_v/F_m, P and Fe ($P<0.05$) in drought exposed plants (Table 3.5). Results of correlation analysis showed that significant negative correlations were found between NPQ, PDW ($P<0.05$) with $\delta^{13}C$ (Table 3.5).

Table 3.4: Mean values and ANOVA results of potassium (K, %), calcium (Ca, %), magnesium (Mg, %), iron (Fe, ppm), zinc (Zn, ppm) and $\delta^{13}c$ (‰) for osmotic stress treatments (T, MPa) in three pistachio rootstocks (R). Values are means ± SE of five replicates.

		P	K	Ca	Mg	Fe	Zn	$\delta^{13}c$
Badami	-0.1	0.27±0.04 a	2.34±0.45 a	2.12±0.16 a	0.4±0.16 a	136±0.3 a	8.78±2.55 a	-29.59±0.53 a
	-0.5	0.09±0.06 b	1.02±0.76 b	1.14±0.54 b	0.24±0.09 ab	134±0.61 a	6.26±4.12 a	-29.55±0.22 a
	-1.0	0.11±0.07 b	1.38±0.71 ab	1.04±0.33 b	0.18±0.04 b	146±0.87 a	7.42±2.9 a	-29.74±0.81 a
	-1.5	0.15±0.05 b	2.06±0.44 ab	1.32±0.18 b	0.2±0.07 b	138±0.81 a	10.72±2.46 a	-29.27±0.59 a
Terebinthus	-0.1	0.08±0.07 a	1.56±0.25 a	1.46±0.18 b	0.56±0.34 a	116±0.31 a	10.38±1.68 b	-28.92±0.71 a
	-0.5	0.12±0.03 a	1.68±0.63 a	1.94±0.3 ab	0.4±0.29 a	136±0.22 a	14.68±4.42 ab	-28.41±0.75 a
	-1.0	0.14±0.12 a	1.42±0.59 a	2.52±0.22 a	0.34±0.11 a	86±0.42 a	14.82±4.28 ab	-28.01±0.31 a
	-1.5	0.22±0.08 a	1.62±0.4 a	2.24±0.53 a	0.6±0.19 a	138±0.62 a	21.96±9.35 a	-28.55±0.38 a
Sarakhs	-0.1	0.42±0.07 a	2.56±0.67 a	2.34±0.34 a	0.26±0.09 a	116±0.29 a	15.0±2.7 a	-30.48±0.86 a
	-0.5	0.27±0.12 ab	2.04±1.05 a	2.02±1.25 a	0.4±0.16 a	232±1.6 a	21.72±13.4 a	-30.46±0.01 a
	-1.0	0.18±0.07 b	2.1±1.16 a	1.9±0.17 a	0.62±0.54 a	204±0.81 a	22.36±3.5 a	-30.47±0.57 a
	-1.5	0.21±0.14 b	1.9±0.27 a	1.54±0.43 a	0.4±0.25 a	100±0.78 a	17.56±2.61 a	-29.81±0.96 a
ANOVA	R	**	*	**	*	ns	**	**
	T	**	ns	ns	ns	ns	ns	ns
	R × T	**	ns	**	ns	ns	ns	ns

Within each column and for each rootstock, means superscripted with different letters are significantly different (** - (P< 0.0 1), * - (P <0.05) and ns- non significant).

Table 3.5: Correlation coefficient analysis results of F_v/F_m, YII, NPQ, qP, PDW, P, K, Ca, Mg, Fe, Zn and $\delta^{13}c$ in three pistachio rootstocks under drought stress conditions. Asterisks indicate significant correlation between two variables (* P <0.05 or ** P< 0.0 1).

	F_v/F_m	YII	NPQ	qP	PDW	P	K	Ca	Mg	Fe	Zn	$\delta^{13}c$
F_v/F_m	1											
YII	0.41**	1										
NPQ	-0.09	-0.30*	1									
qP	0.16	0.83**	0.18	1								
PDW	-0.04	-0.06	0.24	0.06	1							
P	0.20	0.29*	-0.21	0.13	-0.00	1						
K	-0.03	0.27*	-0.08	0.21	0.14	0.52**	1					
Ca	-0.03	-0.13	0.13	-0.10	0.08	0.50**	0.34**	1				
Mg	-0.24	-0.15	0.13	-0.06	0.32*	0.09	0.33*	0.17	1			
Fe	-0.09	-0.08	-0.34**	-0.20	-0.26*	0.07	-0.09	-0.10	0.16	1		
Zn	-0.04	-0.30*	0.05	-0.31*	-0.28*	0.20	0.11	0.29*	0.34**	0.54**	1	
$\delta^{13}c$	0.33*	0.25	-0.42*	0.03	-0.37*	0.39*	-0.01	0.12	-0.03	0.41*	0.33	1

3.3.6 Principal component analysis (PCA)

PCA analysis showed that five main components with eigenvalues > 1 together explained 90.3% of the total variation (appendix 4- chapter 3). PC1 explained 33.96% (Table 3.6) of the total data variation and had highly positive correlation with RDW, LDW, PDW, SDW, PFW and leaf area. The second component (PC2) explained 26.24% of the total variability and correlated positively with YII and qP (both in drought and recovery stages) and F_v/F_m (recovery stage). The third, fourth and fifth dimensions included Zn and shedding, F_v/F_m (drought stage) and Fe which accounted for 18.2%, 6.7% and 5.3% of the total variability, respectively.

Data obtained of PCA of control plants and plants submitted to drought stress were visualized in a biplot analysis of PC1 and PC2, for the three rootstocks (Figure 3.6). The angles between some traits were acute (sharp angle). Therefore, these traits can be classified in four groups with high correlation. Groups 1 and 3 indicated the traits related to photosynthesis rate. Groups 2 and 4 covered traits related to macro and micro elements, respectively and can be considered as groups of plant nutrition (Figure 3.6).

The standard deviation of rootstocks and osmotic drought stress treatments were shown in Figure 3.7. The results from this figure indicated that the distance between Terebinthus under normal (-0.1 MPa) and severe stress (-1.5 MPa) conditions was shorter than two other rootstocks Badami and Sarakhs. These distances in Badami and Sarakhs were fairly similar. Therefore, Terebinthus was less affected by drought stress and can be selected as drought tolerance rootstocks.

Table 3.6: Principle component analysis of measured parameters under different environmental conditions in three pistachio rootstocks.

	Dimension				
Parameters	1	2	3	4	5
F_v/F_m Dr.	-0.058	0.451	0.189	**0.848**	0.025
YII Dr.	0.278	**0.923**	-0.102	0.111	0.015
NPQ Dr.	0.671	-0.489	-0.189	0.288	-0.066
qP Dr.	0.412	**0.871**	-0.188	-0.047	0.118
F_v/F_m Re.	-0.028	**0.71**	0.318	0.553	0.11
YII Re.	-0.439	**0.866**	-0.105	-0.103	0.047
NPQ Re.	0.632	-0.599	-0.076	0.148	0.012
qP Re.	-0.159	**0.711**	-0.302	-0.106	0.101
PFW	**0.886**	0.349	-0.155	-0.156	0.042
PDW	**0.928**	0.262	-0.052	-0.185	0
SDW	**0.908**	0.345	-0.067	-0.176	0.029
LDW	**0.938**	0.251	-0.002	-0.059	0.141
RDW	**0.946**	-0.013	-0.004	0.106	-0.116
Leaf area	**0.891**	-0.387	0.033	0.091	0.118
Shedding	-0.527	-0.151	**-0.803**	0.084	0.075
P	-0.145	0.393	0.774	-0.071	-0.34
K	-0.186	0.596	0.665	-0.291	-0.123
Ca	0.397	-0.107	0.747	-0.011	-0.396
Mg	0.359	-0.181	0.611	-0.029	0.57
Fe	-0.369	-0.04	0.44	-0.135	**0.624**
Zn	-0.138	-0.487	**0.811**	0.052	0.112
Eigenvalue	7.132	5.511	3.828	1.396	1.113
Proportion (%)	33.96	26.242	18.229	6.65	5.3
Cumulative (%)	33.96	60.202	78.431	85.081	90.381

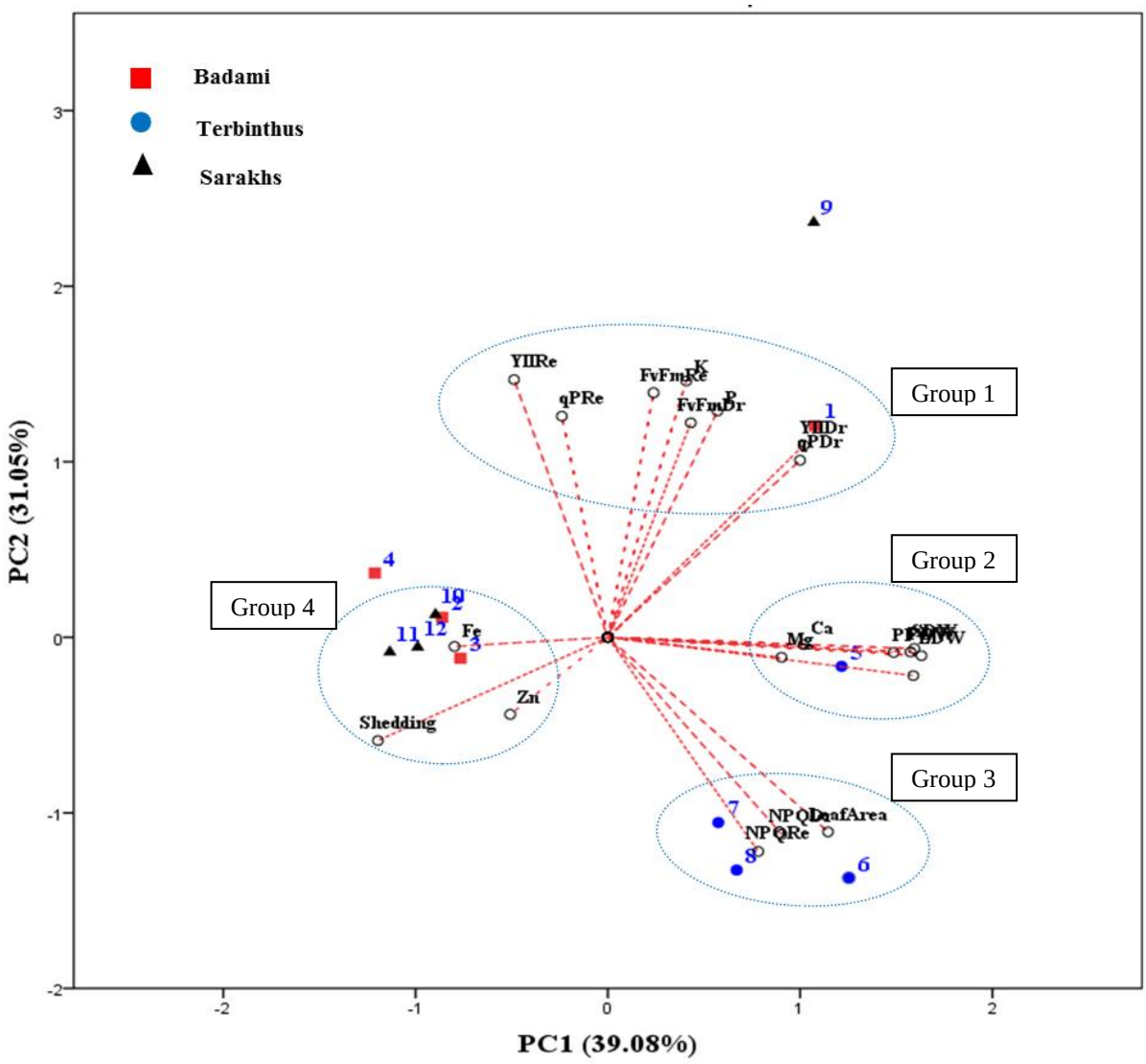


Figure 3.6: Biplot analysis of measured parameters and three pistachio rootstocks under different environmental conditions. Numbers 1 to 4 represent the Badami rootstock under four osmotic drought stress levels: -0.1 (MPa), -0.5 (MPa), -1.0 (MPa) and -1.5 (MPa), respectively; Numbers 5 to 8 represent the Terebinthus rootstock; while Numbers 9 to 12 represent the Sarakhs rootstock both under the same four osmotic drought stress levels.

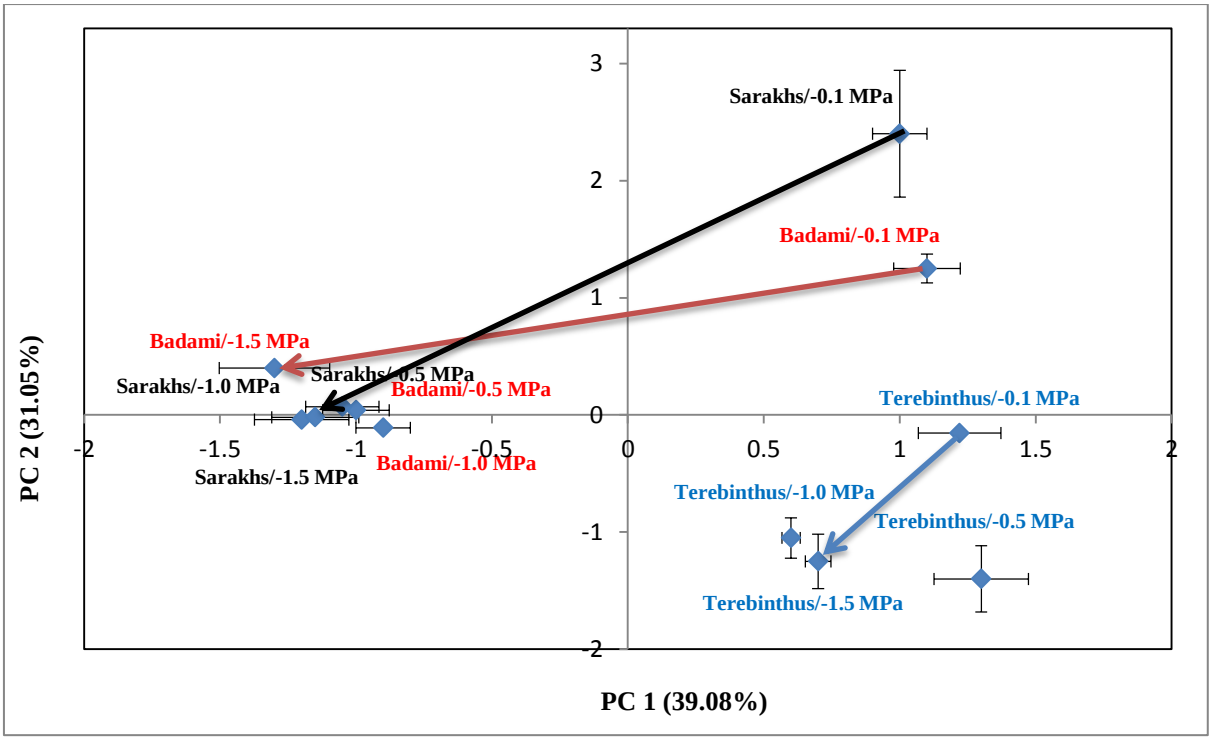


Figure 3.7: Standard deviations (SD) of three pistachio rootstocks (Badami, Terebinthus and Sarakhs) under four osmotic drought stress levels (-0.1 MPa, -0.5 MPa, -1.0 MPa and -1.5 MPa) in Biplot analysis. Horizontal and vertical bars indicate SD for PC1 and PC2 (mean of five replicates ± SD), respectively. Arrows in different colors show the distance between the control (-0.1 MPa) and the severe drought stress (-1.5 MPa) treatments in different rootstocks (red- Badami; blue- Terebinthus and blank- Sarakhs).

3.4 Discussion

Biomass is considered to be an appropriate parameter for evaluation of stress tolerance in many crops (Munns, 2002). In our experiment, osmotic drought stress treatments inhibited dry matter production in three types of pistachio seedlings. When plants were subjected to severe (-1.5 MPa) osmotic drought stress level, biomass (PDW) was found to drop by as much as 57.6 %, 52.8 % and 66.6 % compared to corresponding control plants in Badami, Terebinthus and Sarakhs, respectively. Significant decline of total plant biomass of our pistachio rootstocks in drought treatments compared to control plants is in line with what has been reported for pistachio (Abbaspour et al., 2012b; Habibi and Hajiboland, 2013; Ranjbarfordoei et al., 2000), almond (Rouhi, 2007), and *Ziziphus lotus* (Maraghni et al., 2011). In addition, plant biomass production has been reported to be adversely affected by salt stress treatments in pistachio plants (Abbaspour et al., 2012a; Banakar and Ranjbar, 2010; Fardooei, 2001; Karimi et al., 2009).

Both stomatal and non-stomatal limitations can reduce net photosynthesis (Behboudian et al., 1986). Drought stress limits CO_2 availability following a drought induced stomatal closure. It may lead to reduced photosynthesis (Munns and Tester, 2008) and consequently reduced growth. In addition, non-stomatal factors also affect photosynthesis in severe drought stress conditions (Ogren and Oquist, 1985; Stępień and Kłbus, 2006). To evaluate the direct effect of drought stress on PSII photochemistry, chlorophyll fluorescence parameters were measured in our three pistachio rootstocks.

PSII activity and its regulation are best characterized by YII (Maxwell and Johnson, 2000). In the evaluated pistachio rootstocks, growth inhibition was correlated to a lowered YII. Drought stress levels induced a significant reduction of YII, evidencing decrease in electron transport through PSII in pistachio rootstocks, which is in agreement with the findings of Habibi and Hajiboland (2013). This is also reflected in the decrease of qP in these rootstocks under increasing drought stress compared to the corresponding control plants. These results, however, are in contrast with the findings of Habibi and Hajiboland (2013) who reported that photochemical quenching (qP) was not influenced in drought-stressed pistachio plants under field conditions. The latter parameter gives an indication of PSII ability to reduce primary electron acceptor QA under the applied drought stress as well as of the number of photons used by photochemical reactions per number of absorbed photons (Maxwell and Johnson, 2000). In our study, down-regulation of YII and qP was quite clear during drought and recovery phases.

The qP was hardly affected by increasing drought stress in these rootstocks, because there were no significant differences between the three applied drought stress levels. Photochemical quenching (qP) can contribute to the protection of the photosynthetic apparatus by transferring electrons to O_2 under drought or salt stress (Maxwell and Johnson, 2000). Cornic (1994b) showed that oxygenation of ribulose-1, 5-bisphosphate in C_3 plants can efficiently replace carboxylation when stomata close. We could assume a reduced CO_2 assimilation due to drought stress as dry weight decreased in all rootstocks.

F_v/F_m is generally considered to be a parameter that does not change with stress conditions. A decline in F_v/F_m values indicates serious damage to PSII (Maxwell and Johnson, 2000). Results of our study show that F_v/F_m values were not significantly affected by drought stress, while it significantly decreased during the recovery period. This can be related to drought-exposed plants; the entire photosynthetic apparatus had to be reconstructed during rehydration which can require several hours or even days. Indeed after recovery, the

water content increased in the cell, while the chlorophyll biosynthesis probably did not increase at a similar rate.

Indeed, the decline in F_v/F_m for pistachio rootstocks indicates damage to PSII (Maxwell and Johnson, 2000). Similar results were found with *P. vera* cv. Ahmadaghaii under drought stress (Habibi and Hajiboland, 2013) whereas F_v/F_m was not affected in *P. vera* cv. Badami and cv. Qazvini (Bagheri et al. (2011). Thermal dissipation as evidenced by NPQ was not influenced by different osmotic drought levels for none of the three rootstocks. On the other hand, Ranjbarfordoei et al. (2000) found that thermal dissipation increased under drought stress in *P. mutica* and *P. khinjuk.*

A recovery phase of 14 days was insufficient to restore the plant's full photosynthetic capacity. Romero *et al.* (2004b) found a range of responses in drought-stressed almond, going from partial to rapid recovery of photosynthetic capacity clearly showing varietal differences. Overall, these results are inconsistent with results of Ranjbarfardooei et al. (2002) who reported that chlorophyll fluorescence only significantly changed at the highest level of drought stress (-1.55 MPa) in *Pistacia khinjuk* and *P. mutica*.

Osmotic stress resulted in growth arrest for all rootstocks, and no regrowth was observed after 2 weeks of recovery. Under severe water deficiency, cell elongation of higher plants will be inhibited by an interruption in water flow from the xylem to the surrounding elongating cells (Nonami, 1998). Overall growth reduction observed in our experiment is attributed to a decrease in shoot and leaf biomass, but not to lower root biomass. Decrease in total fresh and dry weight of pistachio rootstocks with increasing drought is in line with those obtained in other research on different pistachio species (Abbaspour et al., 2012b; Ranjbarfordoei et al., 2000; Zhao et al., 2006b).

Root growth is generally less sensitive to drought stress than other biomass components (Hsiao and Xu, 2000). An increase in root/shoot ratio has been proposed as one of the mechanisms involved in the adaptation of plants to drought stress (Turner, 1997). In our study, increasing root/shoot ratios under osmotic stress are only related to a decrease in aboveground biomass (Table 3.3).

In general, *P. terebinthus* maintained the highest plant dry weight (PDW) compared to both other rootstocks (Table 3.3) under drought stress conditions. At the same time, $\delta^{13}C$ in this rootstock was lower compared to that of the other two (data not shown) that can be a good indicator for *P. terebinthus* tolerance to drought conditions (Yousfi et al., 2012b).

Although osmotic stress limits nutrient uptake (Farooq et al., 2009a), an osmotic stress treatment of 14 days seems too short for the observed reduction in leaf nutrients. Nutrient

concentrations have noticeable effects on shoot growth of pistachio seedlings. Afrousheh et al. (2010) reported leaf chlorophyll content was greatly influenced by the deficiency of various nutrient elements. All the fractions of chlorophyll of treated seedlings, particularly N-deficient and Mn-deficient seedlings, reduced considerably. This was because of inadequate supply of N for chloroplast protein synthesis. N deficiency had a noticeable effect on the growth of seedlings particularly with regard to shoot growth. The affected plant has little or no ability to manufacture carbohydrates through photosynthesis and may die unless the cause of its chlorophyll insufficiency is treated. Shanker Dubey (2005) represented deficiency of different nutrient elements in leaves presumably due to earlier break of chloroplast, older leaves, decreasing of chlorophyll and finally decrease of photosynthesis. Also limiting of nutrient elements due to starch synthesis and accumulation of sugar had a limiting effect on photosynthetic chemical reactions. Thus, drought-induced P, K, and Ca loss in Badami and Sarakhs, could be another factor that induced growth arrest in these rootstocks.

Concentrations of Ca and Zn significantly increased by increasing drought stress in Terebinthus (Table 3.4). On the other hand, this rootstock had the highest values for growth parameters. It has been noted that calcium could participate in the regulating mechanism in plants adjusting to adverse conditions such as drought (Bowler and Fluhr, 2000), and salt stress (Cramer *et al.*, 1985). Inorganic ions for osmotic adjustment are mainly Na^+, K^+, Ca^{2+}, and Cl^-. Inorganic ions make great contribution in osmotic adjustment by ion transport processes with related ion antiporters and ion channels (Chen and Jiang, 2010). Also, cytosolic Ca^{2+} is a second messenger for a number of extracellular signals in plants and more specifically calcium serves as a second messenger when ABA triggers the closing of guard cells. ABA is produced under water stress conditions in vegetative tissues and controls stomatal closure and gene expression related to dehydration tolerance (Finkelstein and Rock, 2002; Rock *et al.*, 2009). Therefore, increasing calcium concentration in Terebinthus rootstock under drought stress condition regulates plant water level by controlling transpiration rate; and act as an inorganic ion for osmotic adjustment.

Zinc is required to make auxin, the plant hormone responsible for cell elongation and growth. Zinc is also needed in the biosynthesis of chloroplasts, the leaf component that contains chlorophyll (Beede *et al.*, 2005). Therefore, these two elements (Ca and Zn) can play important role to maintain growth and induce tolerance under drought stress conditions in pistachio Terebinthus.

When the nutrient concentration in plant tissue is low, growth is reduced. An increase in nutrient availability is directly related to an increase in growth or yield. Table 3.7 shows adequate and critical levels of nutrient elements in pistachio (*P. vera*) leaves. According to critical levels of nutrient elements mentioned in Table 3.7, concentrations of P, K and Ca in severe drought stress treatments were not lower than critical levels of these macronutrients. Thus, these nutrient elements could have a positive effect on drought tolerance in pistachio plants.

Findings of this study on leaf $\delta^{13}C$ for drought stress treatments are in contrast with the results of Hokmabadi et al. (2005), who reported carbon isotope discrimination (Δ) decreases with increasing levels of salinity in leaves, stems and roots of pistachio seedlings. Our results on rootstocks are also in contrast with those of Hokmabadi et al. (2005), as we observed significant differences among pistachio rootstocks (Table 3.4). However, Pascual et al. (2013) showed leaf carbon isotope ratio was not significantly affected by either irrigation or nitrogen treatments among peach (*Prunus persica*) rootstocks.

Table 3.7: Adequate and critical levels of nutrient elements in pistachio (*P. vera*) leave (Ferguson et al, 2005).

Elements	Chemical symbol	Unit	Adequate levels in dry matter	Critical level
Nitrogen	N	%	2.2- 2.5	1.80
Phosphorus	P	%	0.14 -0.17	0.14
Potassium	K	%	1.8- 2.0	1.60
Calcium	Ca	%	1.3- 4.0	1.30
Magnesium	Mg	%	0.6- 1.2	0.60
Iron	Fe	ppm	150	100
Manganese	Mn	ppm	30- 80 ppm	30
Zinc	Zn	ppm	10-15.0	7
Cupper	Cu	ppm	6-10.0	10
Boron	B	ppm	150- 250	90
Sodium	Na	%	-	0.10
Chlorine	Cl	%	0.1 - 0.3	-

There were significant positive relationships between $\delta^{13}C$ and F_v/F_m, P and Fe ($P<0.05$). In addition, results show that there were significant negative relationships between carbon isotope compositions ($\delta^{13}C$) and NPQ and PDW ($P<0.\ 05$) parameters (Table 3.5) during the

experiment. Based on these correlations, carbon isotope composition is a good indicator for pistachio rootstocks to screen the genotypes.

According to data of Table 3.6, the PC1 can be named as a biomass component. In other words, this component was able to separate the rootstocks with high biomass production under different drought stress conditions. The second component (PC2) indicates the photosynthesis. It means with apply some chlorophyll fluorescence parameters (YII and qP) that related to photosynthesis, this component was able to separate the pistachio rootstocks with high photosynthesis. Thus, selection of rootstocks that have high PC1 and high PC2 are suitable for different stress conditions because these rootstocks have a high growth along with high photosynthesis rate.

From the biplot, rootstock Sarakhs (9), Badami (1) and Terebinthus (5) with high PC1 had high photosynthesis rate, respectively under non stress condition. Biplot depicted that Terebinthus (6 and 7) as compared with Badami (2 and 3) and Sarakhs (10 and 11) represented higher PC1 under moderate drought stress condition (-0.5 and -1.0 MPa) and was selected as tolerant rootstock with high growth in these conditions. Under severe stress condition (-1.5 MPa), Terebinthus (8) with higher PC1 values was selected as tolerant rootstock compared with Badami (4) and Sarakhs (12). Generally, these results of biplot identified Terebinthus and Sarakhs as the most tolerant and susceptible rootstock under different stress conditions, respectively.

The results of PCA and biplot analysis selected RDW, LDW, PDW, SDW, PFW,YII, qP, F_v/F_m and shedding as the suitable indicators for screening and classification of pistachio rootstocks under different stress conditions. These results also indicated that rootstocks belonging to the *P. vera* species (Badami and Sarakhs) with close distance were located in the same group under drought stress conditions, while *P. terebinthus* was located in the other group. Terebinthus with highest PC1 under drought stress conditions (6, 7 and 8) was identified as the most tolerant rootstock. The biplot revealed that plant nutrients including Fe, Zn, Ca and Mg cannot be ideal for evaluating genetic diversity and screening pistachio rootstocks under normal and stress conditions.

3.5 Conclusion

Pistachio trees are considered to be drought-tolerant, yet the applied osmotic drought stress conditions induced significant reductions in chlorophyll fluorescence parameters (YII and qP), decline in fresh and dry plant weights and reduction in leaf nutrient contents. The

period of recovery from the applied stress was longer than expected, as after two weeks of recovery negative effects of the stress were still evident at chlorophyll fluorescence parameters.

YII could be used to distinguish the different responses to drought stress in the studied pistachio rootstocks. Consequently, light-adapted responses could be considered as an early indicator of drought-induced disturbances in pistachio. As YII remained to be affected after a drought stress period, this parameter has a potential to be an early and non-destructive tool to screen pistachio rootstocks for drought stress.

Terebinthus rootstock had better tolerated the applied drought stress as shown by the better growth parameters performance in drought stress conditions and the lower carbon isotope composition (more negative) value when compared with Badami and Sarakhs rootstocks. Based on increase in δ^{13}c, Badami is moderately tolerant to drought stress, whereas Sarakhs seems more sensitive to the applied drought stress. Resistance of Terebinthus with highest PC1 under drought stress conditions was identified by Biplot presentation. However, further research in field conditions is needed to confirm this survey's research results**.**

Chapter 4. **Osmotic stress affects physiological responses and growth characteristics of three pistachio (*Pistacia* vera L.) cultivars**

Adapted from:

Esmaeilpour, Ali; Van Labeke, Marie-Christine; R. Samson, P. Boeckx; Van Damme, Patrick. 2015. Osmotic stress affects physiological responses and growth characteristics of three pistachio cultivars. *Acta Physiologiae Plantarum* 37(6):1-14.

Esmaeilpour, A**.** and P. Van Damme. 2014. Evaluation of Seed Soaking Times on Germination Percentage, Germination Rate and Growth Characteristics of Pistachio Seedlings. The 29th international horticultural congress (IHC2014). Brisbane, Australia.

Esmaeilpour, A**.,** M. C. Van Labeke, R. Samson and P. Van Damme. 2014. Chlorophyll fluorescence as a tool for evaluation of drought stress tolerance in Iranian pistachio cultivars. International conference on research on food security, natural resource management and rural development (Tropendag 2014). September 17-19. Prague, Czech Republic.

4.1 Introduction

Drought is a common and serious problem to plants in arid or semi-arid areas. Plants have developed different morphological, physiological and biochemical mechanisms to withstand drought stress. Drought stress limits plant growth and development as well as a wide range of physiological processes such as photosynthesis (Anjum et al., 2011; Blum, 2005; Farooq et al., 2009b) and causes a higher rate of impairment than any other environmental factors (Cechin et al., 2008; Shao et al., 2009). Plant responses to drought stress depend on timing, severity, and length of the drought event (Anjum et al., 2011; Chaves et al., 2002; Praba et al., 2009). Maintenance of cell turgor plays an important role in regulating the carbon balance of plants. Water stress greatly suppresses cell expansion and cell growth as a result of the low turgor pressure (Shao et al., 2008). Turgor pressure maintenance allows stomata to remain open, photosynthesis to continue and growth to be uninterrupted (Pessarakli, 1994). Furthermore, under progressive drought stress, electron transport will be impaired followed by an enhanced production of reactive oxygen species (Cornic and Fresneau, 2002; Cruz de Carvalho, 2008; Gnaana Saraswathi and Paliwal, 2011). Moreover, a reduced leaf area and decreased transpiration rate are found to occur under drought stress (Dong and Zhang, 2000; Liu and Stützel, 2004). One of the most common stress responses in plants is an enhanced biosynthesis of different types of compatible organic solutes (Serraj and Sinclair, 2002). Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. Generally, they protect plants from stress trough different ways, including contribution to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Bohnert and Jensen, 1996; Yancey *et al.*, 1982). Proline is one of the most common compatible solutes in drought-stressed plants (Ashraf and Foolad, 2007; Matysik et al., 2002; Yoshiba *et al.*, 1997). Proline is not only an important molecule in redox signaling, but also an effective hydroxyl radical scavenger (Miller et al., 2010). Proline also protects cell membranes against the negative effects of ROS (Claussen, 2005; Matysik et al., 2002). The osmo-protective function of proline accumulation under drought stress is well-documented (Blum, 2005; Matysik et al., 2002; Miller et al., 2010). Enhanced synthesis of proline under drought or salt stress is a mechanism to alleviate cytoplasmic acidosis and to maintain NADP at values compatible with metabolism (Miller et al., 2010; Saradhi, 1991). An additional NADPH advantage of the refilling of NADP supplied by proline synthesis may

be to support redox cycling, which is especially important in plant antioxidant defense mechanisms during stress (Babiychuk *et al.*, 1995; Miller et al., 2010).

Drought stress was evaluated for *P. vera* Kerman grafted onto three different pistachio rootstocks. Grafting onto hybrid rootstock (UCB#1) and *P. terebinthus* resulted in a higher growth reduction compared with *P. atlantica* under drought stress (Gijón et al., 2010). However, physiological adaptations of pistachio to drought stress have only been studied to a limited extent.

Akbari, Kaleghochi and Ohadi pistachio (*P. vera*) cultivars are the most common cultivars in Iran (Esmaeilpour and Khezri, 2006; Panahi et al., 2002; Sheibani, 1995) which are grown under different environmental conditions. Yet, their physiological responses to drought stress have not been studied. The aim of this study was to evaluate the effects of osmotic drought stress and subsequent recovery on the physiological performance of these three cultivars. Plant responses were assessed by evaluating leaf water potential, photosynthesis, chlorophyll fluorescence, chlorophyll pigments, proline, nitrogen and growth characteristics. It is hypothesized that photosynthetic performance and proline accumulation could be used as indices for screening drought-tolerant genotypes.

4.2 Materials and Methods

4.2.1 Plant material and experimental set-up

This study was carried out in a glasshouse at the Faculty of Bioscience Engineering, Ghent University (51°3' N, 3°42' E). Certified seeds of three pistachio cultivars (Figure 4.1), *Pistacia vera* L. Akbari (AK), Kaleghochi (KA), and Ohadi (OH), were obtained from the Iranian Pistachio Research Institute (IPIR), Rafsanjan, Iran. Seeds were first soaked in water for 12 hours and then pre-treated for 20 minutes with 0.01% captan (N-trichloromethyl-thio-tetrahydro phthalimide) solution, a broad-spectrum fungicide (Panahi et al., 2002). All seeds were sown in 4-L pots containing sand and organic material in June 2011. Plant management was done according to good agricultural practices (including soil preparation, planting, irrigation, thinning, staking, pruning, pest and disease control) during the growing seasons (Panahi et al., 2002; Sheibani, 1995). In March 2012, seedlings were transplanted to 5-L pots filled with vermiculite. Transplanted 1-year-old seedlings were grown hydroponically in a controlled glasshouse environment using standard Hoagland solution (Picchioni et al., 1991) for fertigation. Plants were irrigated using a circulating system as shown in Figure 4.2. In order to prevent adverse effects of PEG on oxygen availability, the nutrient solution was

continuously aerated. Temperature and relative humidity in the glasshouse ranged between 22-27ºC and 50-71% RH respectively, while daily mean and maximum light intensity was 212 µmol m^{-2} s^{-1} and 862 µmol m^{-2} s^{-1} PAR (quantum sensor SKP215, Skye at plant canopy and connected to a data logger, type DL3000, Delta-T, UK).

Figure 4.1: Seeds of different pistachio cultivars (A, Akbari; B, Kaleghochi and C, Ohadi) to study the response of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

In May 2012, osmotic drought treatments were applied and consisted of a control (osmotic potential of the nutrient solution (Ψ_s= -0.10 MPa) and two drought stress levels (moderate stress, $\Psi_{s=}$ -0.75 MPa, severe stress, $\Psi_{s=}$ -1.5 MPa) using Polyethylene glycol (PEG) 6000. Drought stress levels were chosen according to the literatures about *Pistacia* species. Concentration of PEG 6000 needed to obtain the respective drought stress levels was determined following (Michel and Kaufmann, 1973) and adjusted/controlled using an osmometer manually and weekly (Fiske One-Ten, Fiske Associates, Howard, USA). For a description of the PEG concentration, see § 3.2.1.

The three solutions were oxygenated using an electric pump. Each treatment was applied in three cultivars with 9 plants as replications. Stress levels were maintained for two weeks; then all solutions were replaced by the control treatment, and this level was maintained for two recovery weeks.

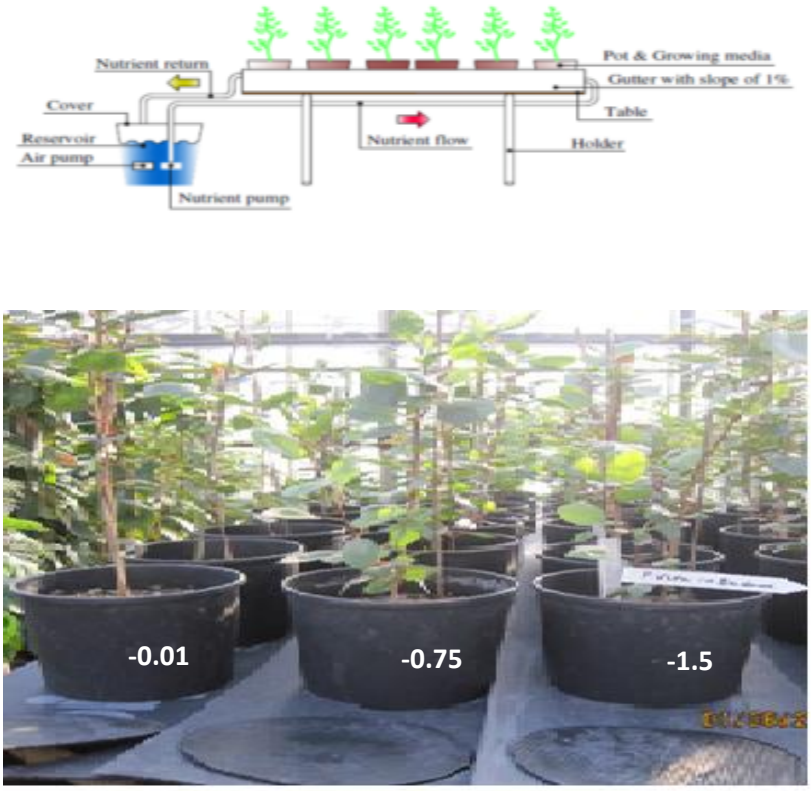


Figure 4.2: Experimental setup to study the response of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

4.2.2 Measurements

4.2.2.1 Leaf water potential

Midday leaf water potential (Ψ_l) was measured after 14 days of drought stress and after 14 days of recovery, this means in 6 replicates (plants) per each cultivar. Leaf water potential measurements were performed on a fully expanded leaf by using a pressure chamber (Model 1000, PMS Instrument Company, Albany, OR, USA) and using nitrogen gas for pressurization (Kramer and Boyer, 1995) (Figure 4.3, A and B) between 11 am and 1 pm. Air temperatures were on average 26.8°C and 29.4°C and relative humidity were 49.6 % and 37.6 % on measuring days after drought and recovery stages, respectively. Climate conditions (temperature (°C), relative humidity (%) and PAR (µmol/m².s)) were recorded during the experiment and are shown in (Figure 4.4 A, B and C).

4.2.2.2 Photosynthesis measurements

Gas exchange measurements were done on the eighth leaf from the apex using a CO_2 and H_2O infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, USA) (Figure 4.3, C and D) in five replicates (plants) per cultivar. Net photosynthetic rate (*Pn*), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and leaf transpiration rate (E) were measured after 14 days of drought stress and after 14 days of recovery. All measurements were made under standard environmental conditions (light intensity of 1500 µmol.m^{-2}s^{-1}, ambient CO_2 concentration (C_a) of 400 µmol CO_2 mol^{-1}, leaf temperature (T_l) = 26.9ºC, leaf vapor pressure deficit (VPD_l) = 1.1-2.2 kPa) between 10 am and 3 pm. The chamber temperature of the fluorescence head was set to match the actual temperature measured in the treatment environment at the start of the measurements (25°C). Every plant measurement lasted for 4-5 minutes. Intrinsic water use efficiency (WUE) was estimated as *Pn*/*gs* (Mediavilla *et al.*, 2002).

4.2.2.3 Chlorophyll fluorescence measurements

For a description of the chlorophyll fluorescence measurements, see § 3.2.2.1

Figure 4.3: Measurement of leaf water potential (A and B), and gas exchange parameters (C and D) of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

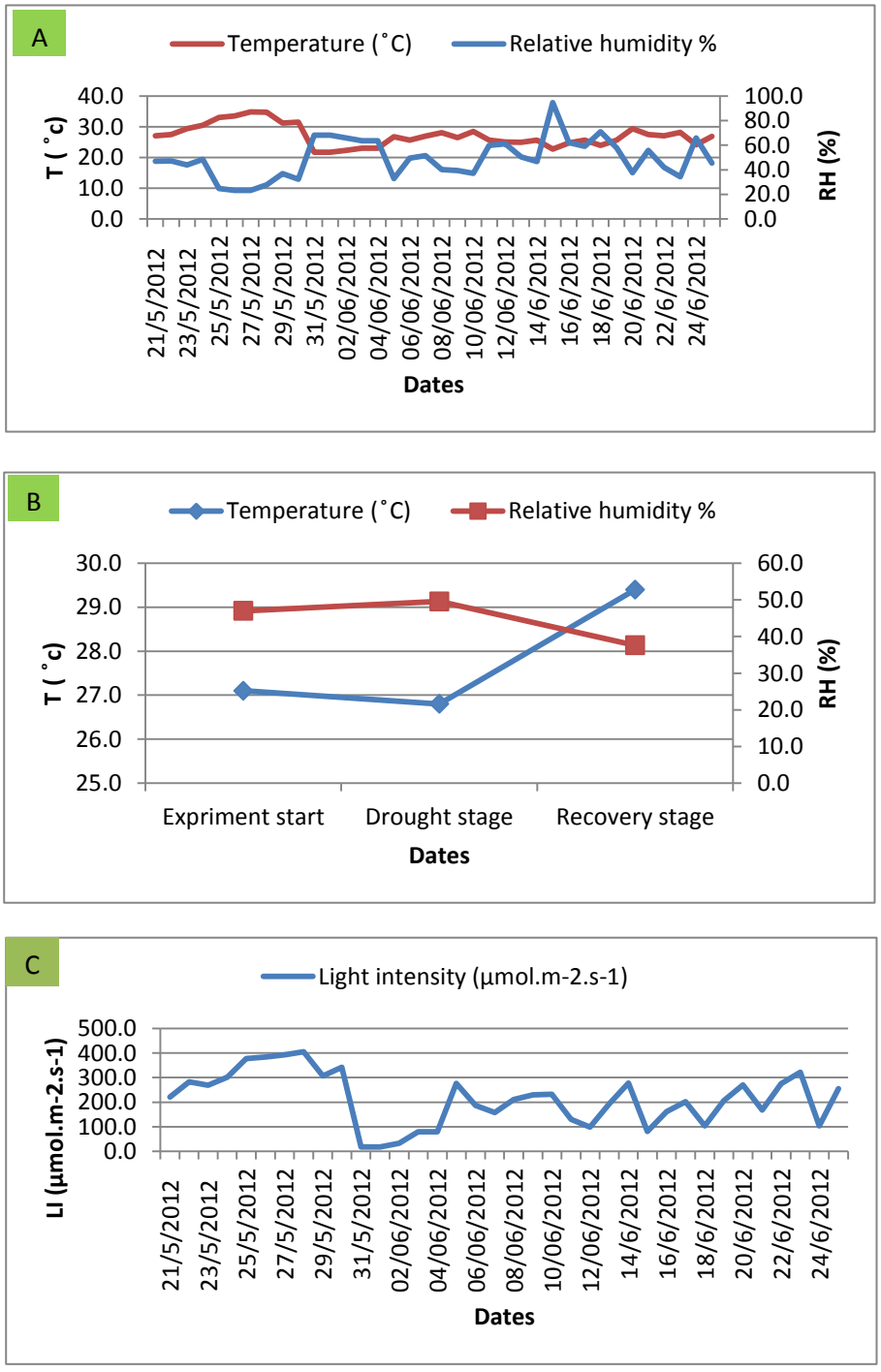


Figure 4.4: Average temperature and relative humidity (means) during the experiment (A), and at the start of drought and recovery stages (B) and photosynthetic active radiation (PAR) during the experiment (C) in the glasshouse of faculty of Bioscience engineering, Ghent, Belgium.

4.2.2.4 Chlorophyll content

Leaf material (150 mg fresh weight) was ground with liquid nitrogen. After extraction in 80% acetone, samples were shaken and then stored at -20ºC for 24 hours. Pigments were measured spectrophotometrically (UVIKON$_{XL}$, BIO-TEK Instrument, USA) (Figure 4.5, A & B), in nine replicates (plants). Chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), and carotenoids (Car) (µg g^{-1} FW) were calculated according to Lichtenthaler (1987):

Chl a = 12.25 $A_{663.2}$ - 2.79 $A_{646.8}$,

Chl b = 21.5 $A_{646.8}$ - 5.1$A_{663.2}$, and

Car = (1000 A_{470} - 1.82 Chl a - 85.02 Chl b) /198.

4.2.2.5 Proline content

For proline two leaves per plant were collected after two weeks of drought stress and two weeks of recovery, in 6 replicates (plants). Leaf samples (±500 mg FW) were extracted with 3% aqueous sulfosalicylic acid. Concentration of free proline was determined by the acid ninhydrin method according to Bates *et al.* (1973) and measured spectrophotometrically at λ = 520 nm (Nanoquant Infinite M200, TECAN Austria GmbH, 5082 Grodig, Austria) (Figure 4.5, C & D).

4.2.2.6 Leaf nitrogen content

Dried leaf samples were ground by a grinder (ZM200, Retsch, Germany). Three plants per treatment were analyzed with an elemental analyzer (ANCA-SL, PDZ-Europe, Northwick, UK) coupled to an isotope ratio mass spectrometer (model 20–20, Sercon, Crewe, UK) to measure total C and N content (Roobroeck *et al.*, 2010).

Figure 4.5: Measurement of chlorophyll contents (A and B), and proline contents (C and D) of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

4.2.2.7 Plant growth parameters

At the end of the second recovery week, all plants were harvested. Fresh weight of leaves, shoots and roots was determined (Figure 4.6, A- C) with an electronic precision balance ± 0.1 mg (Mettler Toledo PB602-L, Greifensee, Switzerland), in nine replicates (plants) per each cultivar. Dry weight of the plant fractions was determined after drying at 85°C for 72 hours (Jouan laboratory oven, UK).

Plant height of each plant was measured from the base (the point where roots separate from the stem) at the beginning (H_1) and end of the experiment (H_2) with a ruler (± 0.1 cm). The stem elongation (SEL) was calculated using the following equation:

$SEL= H_2-H_1$ (5.1)

Where; H_1 is stem height before imposition of drought stress and H_2 is stem height at the end of the experiment. Stem diameter just above soil surface was determined with a digital

caliper in the same way. As stem elongation (Plant height) that mentioned earlier, leaf number was not counted during this experiment.

Figure 4.6: Measurement of fresh and dry weights (A, B and C) of different parts of pistachio seedlings to water deficit induced by PEG at varying water potentials in nutrient solution under glasshouse conditions.

4.2.3 Statistical analysis

The experiment was designed as a randomized complete block design (RCBD) with nine treatment levels (three cultivars* three drought stress levels) and nine replications. A two-way analysis of variance was used to test for effects of drought treatments and cultivars. One-way ANOVA to test treatments by cultivar was also performed. Means were compared using Tukey's HSD test ($P<0.05$). Correlations between parameters were calculated using Pearson's correlation method. Multivariate analysis of variance (MANOVA) was conducted for all measured parameters in chapters 4 and 5. A normality test was conducted using descriptive statistical parameters and a homogeneity test was done to see diverse data using Levene's test of equality of error variance by SPSS software. All analyses were performed in SPSS version 20 (IBM Corporation, USA).

4.3 Results

4.3.1 Multivariate analysis of variance (MANOVA)

The MANOVA analysis (Table 4.1) revealed no significant differences among three cultivars. There are significant differences among three drought stress conditions (treatments) by considering all parameters. Interaction effects of cultivar * treatment was significant, indicating different responses of genotypes to water stress condition and possible selection of drought tolerant genotypes under water deficit.

Table 4.1: Multivariate analysis of variance (MANOVA) for all measured parameters under different environmental conditions in three pistachio cultivars.

Effect	Test	Value	F	Hypothesis df	Error df	Sig.
Cultivar	Roy's Largest Root	45.30	1.46	62	2	0.492
Treatment	Roy's Largest Root	530.23	17.10	62	2	0.057
Cultivar * Treatment	Roy's Largest Root	162.23	10.47	62	4	0.016
Block	Roy's Largest Root	6069.6	783.17	62	8	< 0.001

4.3.2 Plant water relations

Midday leaf water potential decreased significantly with increasing drought stress (Figure 4.7, top). The largest decrease was found for $\Psi_{s=}$ -1.5 MPa where leaf water potentials ranged between -1.05 MPa and -1.13 MPa, respectively for Akbari and Ohadi. Two weeks of recovery still resulted in a more negative leaf water potential for Kaleghochi and Ohadi at the highest stress level, compared with control plants (Figure 4.7, bottom).

4.3.3 Gas exchange parameters and WUE

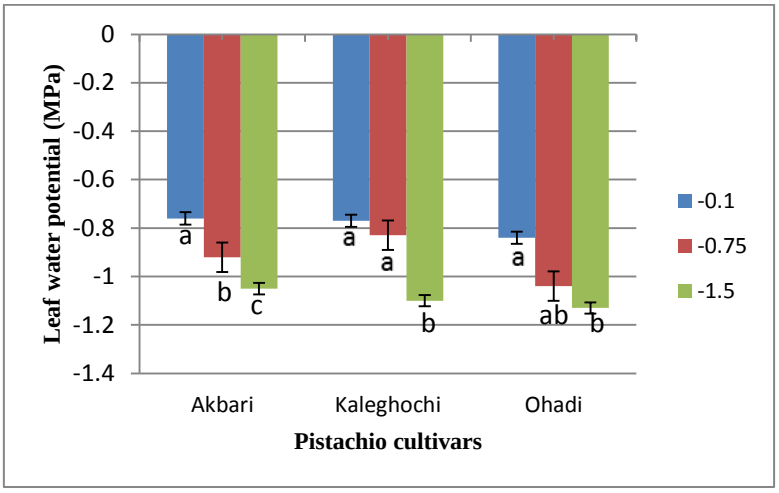
Drought stress significantly decreased *Pn*, *gs*, C_i, and E, whereas WUE increased for the three cultivars (Table 4.2). Effects remained visible after a recovery phase of 14 days. These parameters showed no significant differences between drought stress levels after rewatering.

Pn was reduced by 60%, 33% and 34 % for Akbari, Kaleghochi, and Ohadi, respectively, after two weeks at -1.5 MPa compared with control plants (Table 4.2), while stomatal conductance was reduced by 50%, 66%, and 52% for Akbari, Kaleghochi, and Ohadi, respectively, after two weeks at -1.5 MPa (Table 4.2). Leaf transpiration rates also decreased significantly under severe drought stress compared with their respective controls for Akbari, Kaleghochi, and Ohadi (Table 4.2).

Ohadi better maintained a high *Pn*, C_i, and E after 2 weeks of severe osmotic stress compared with the other two cultivars ($P < 0.5$) (Table 4.2). Intrinsic water use efficiency increased significantly when the solution's osmotic potential was decreased, although for Ohadi, the highest WUE was obtained after two weeks at -0.75 MPa (Table 4.2). After two weeks of recovery, *Pn*, *gs*, C_i, and E were still significantly lower than for the control, while WUE was higher (Table 4.2).

In short, effects of different drought stress treatments were significant on *Pn*, *gs*, C_i, E, and WUE in both drought and recovery stages ($P < 0.01$). No significant interactions between cultivar and treatments were found for the studied parameters under drought or after rewatering (Table 4.2). Figure 4.8 shows the response of C_i/C_a to decreasing *gs* under drought stress. The lowest value of C_i/C_a (0.16) occurred at *gs* of about 0.03 mol $m^{-2}s^{-1}$.

DROUGHT



RECOVERY

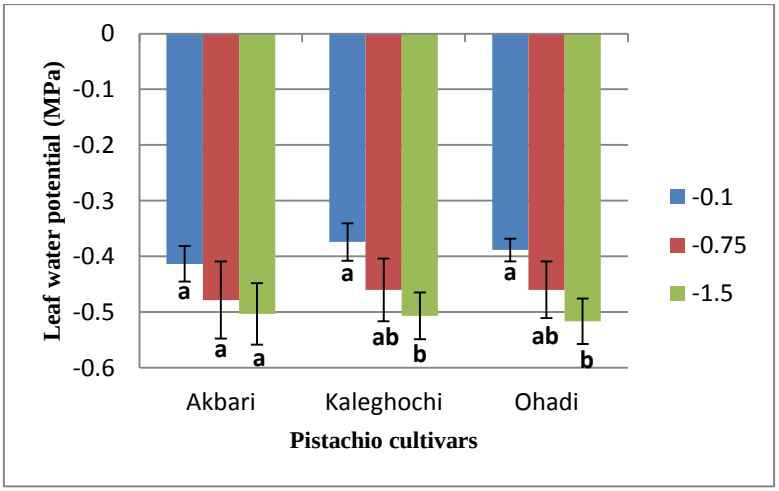


Figure 4.7: Midday leaf water potential (MPa) of three pistachio cultivars subjected to a 14 d-period of drought osmotic stress (top) and a 2 week-period of recovery (bottom). Each value is the mean of six replicates. Different letters indicate significant differences between treatments by Tukey's HSD test ($P < 0.05$).

4.3.4 Chlorophyll fluorescence

Increasing osmotic drought stress decreased F_v/F_m in Akbari, yet no effect was observed for the other cultivars. No significant differences in YII under increasing stress were observed for the three cultivars, while qP decreased in drought-stressed Akbari and Ohadi (Table 4.3). During recovery, effects of cultivar and interaction effects were non-significant for all chlorophyll fluorescence parameters (Table 4.3). Overall, the effect of drought stress on NPQ was not significant after two weeks of drought, although NPQ decreased for the severe stress level in Akbari (Table 4.3). Significant effects were found in the recovery stage ($P < 0.01$); NPQ increased in Kaleghochi and Ohadi (Table 4.3). An overall negative effect for electron transport rate (ETR) was found after 2 weeks of drought stress whereas 2 weeks of recovery were insufficient to restore electron transport rate (Table 4.3).

Table 4.2: Gas exchange parameters in three pistachio cultivars (C) after a 14 d-period of osmotic stress (T, MPa) and a 2 week-period of recovery. Values of *Pn* (µmol $m^{-2}s^{-1}$), *gs* (mol H_2O $m^{-2}s^{-1}$), C_i (µmol CO_2 mol^{-1}), E (mmol (H_2O) $m^{-2}s^{-1}$) and WUE (*Pn* /*gs*). Values are means ± SE of five replicates.

		DROUGHT					RECOVERY				
C	T	*Pn*	*gs*	C_i	E	WUE	*Pn*	*gs*	C_i	E	WUE
Akbari	-0.1	15.2± 3.5 a	0.25±0.07 a	254.0±20.8 a	4.0±0.9 a	78.2±12.6 c	19.0±1.1 a	0.46±0.08 a	285.2±6.5 a	3.5±0.3 a	47.0±4.8 b
	-0.75	7.30± 0.1 b	0.06±0.00 b	217.0±9.8 b	1.6±0.1 b	101.9±4.6 b	11.1±1.3 b	0.17±0.05 b	231.4±19.0 a	1.6±0.3 b	89.6±12.8 a
	-1.5	6.0± 1.4 c	0.07±0.02 b	206.9±19.2 c	1.3±0.3 c	110.7±12.4 a	11.8±0.5 b	0.17±0.04 b	232.7±20.1 a	1.6±0.2 b	87.8±12.8 a
Kaleghochi	-0.1	11.2± 3.4 a	0.18±0.06 a	272.2±7.6 a	3.1±0.8 a	67.6±3.7 b	17.8±1.5 a	0.37±0.06 a	273.0±8.5 a	3.1±0.4 a	55.7±5.9 b
	-0.75	7.9±1.2 b	0.07±0.01 b	191.7±21.9 c	1.6±0.3 b	120.6±13.6 a	11.3±0.9 b	0.21±0.04 ab	268.3±12.5 a	2.0±0.3 ab	65.9±7.9 b
	-1.5	7.4±0.7 c	0.06±0.01 c	194.4±13.7 b	1.4±0.1 c	115.7±7.9 a	9.3±1.1 b	0.10±0.03 b	206.6±22.4 b	1.2±0.2 b	133.0±31.8 a
Ohadi	-0.1	14.6±1.3 a	0.19±0.02 a	251.7±13.7 a	3.5±0.3 a	77.4±2.7 c	18.9±1.7 a	0.43±0.06 a	277.4±11.1 a	3.4±0.4 a	51.8±8.1 a
	-0.75	6.5±1.7 c	0.06±0.02 c	199.2±8.9 c	1.3±0.4 c	115.8±5.9 a	7.9±0.5 b	0.10±0.02 b	241.2±14.7 a	1.2±0.2 b	86.3±9.2 a
	-1.5	9.6±0.3 b	0.09±0.01 b	208.4±10.9 b	2.0±0.1 b	107.9±4.9 b	11.4±1.1 b	0.16±0.03 b	229.2±21.9 a	1.6±0.2 b	90.6±14.1 a
ANOVA	C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	T	**	**	**	**	**	**	**	**	**	**
	C x T	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Within each column and for each cultivar, means superscript with different letters are significantly different (ANOVA analysis: ** - $P< 0.0 1$,*- $P<0.05$ and ns- none significant). Degrees of freedom for cultivars (C), treatments (T) and interaction effects (C*T) are; 2, 2 and 4, respectively.

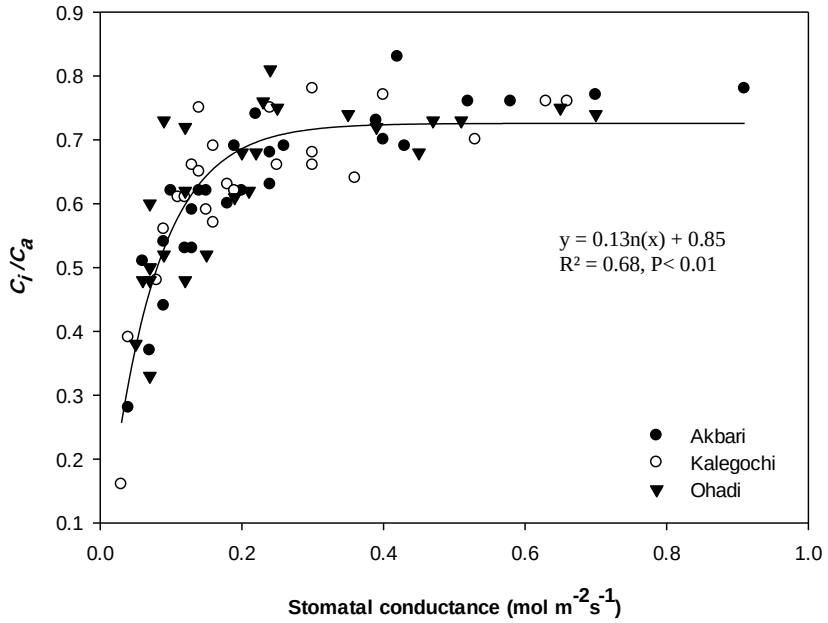


Figure 4.8: Changing ratio of C_i/C_a as a function of stomatal conductance for three pistachio cultivars under increasing drought stress. Each symbol represents a value for every cultivar as given in Table 4.2; the line represents the following linear relationship: y= 0.13n(x) + 0.85; R^2= 0.68; n=27; $P < 0.01$.

4.3.5 Chlorophyll and carotenoid contents

Chl *a* and Chl *b* decreased after 2 weeks of drought stress, though differences among drought stress levels were not significant (Table 4.4). No significant reduction of Car was noted under drought stress (Table 4.4). During recovery, Chl *a*, Chl *b* and Car were still significantly lower for the drought stress treatments (Table 4.4). No cultivar effects and no interaction between cultivar and treatment were found for the pigments during neither drought nor recovery (Table 4.4).

Table 4.3: Changes in chlorophyll fluorescence parameters in three pistachio cultivars (C) after a 14 d-period of osmotic stress (T, MPa) and a 2 week-period of recovery. Values are means ± SE of nine replicates.

		DROUGHT					RECOVERY				
C	T	F_v/F_m	YII	NPQ	qP	ETR	F_v/F_m	YII	NPQ	qP	ETR
Akbari	-0.1	0.79±0.00 a	0.20±0.01 a	1.88±0.07 a	0.49±0.02 a	38.1±2.2 a	0.78±0.00 a	0.29±0.01 a	1.60±0.10 a	0.64±0.02 a	54.1±2.9 a
	-0.75	0.76±0.01 b	0.18±0.01 a	1.94±0.06 a	0.50±0.03 a	33.7±1.5 a	0.75±0.01 b	0.18±0.01 b	1.82±0.06 a	0.51±0.03 b	33.3±3.3 b
	-1.5	0.76±0.01 b	0.17±0.02 a	1.41±0.13 b	0.39±0.05 b	32.7±3.0 a	0.74±0.01 b	0.17±0.02 b	1.60±0.08 a	0.42±0.03 b	31.2±2.7 b
Kaleghochi	-0.1	0.77±0.01 a	0.32±0.13 a	1.78±0.06 a	0.47±0.04 a	39.6±5.4 a	0.77±0.01 a	0.30±0.02 a	1.50±0.11 b	0.69±0.01 a	57.8±2.9 a
	-0.75	0.75±0.01 a	0.18±0.02 a	1.75±0.08 a	0.45±0.04 a	36.0±3.0 a	0.77±0.01 a	0.20±0.02 b	1.78±0.04 a	0.55±0.03 b	38.6±3.3 b
	-1.5	0.76±0.01 a	0.17±0.01 a	1.78±0.08 a	0.44±0.02 a	32.7±2.5 a	0.76±0.01 a	0.17±0.02 b	1.82±0.05 a	0.47±0.05 b	31.5±1.4 b
Ohadi	-0.1	0.78±0.01 a	0.36±0.01 a	1.80±0.14 a	0.54±0.04 a	39.9±3.2 a	0.77±0.01 a	0.27±0.01 a	1.50±0.11 b	0.60±0.02 a	51.8±1.8 a
	-0.75	0.77±0.00 a	0.15±0.01 a	1.83±0.07 a	0.39±0.03 b	32.0±2.5 ab	0.74±0.01 b	0.17±0.01 a	1.85±0.10 a	0.49±0.03 b	34.2±2.1 b
	-1.5	0.76±0.01 a	0.17±0.01 a	1.94±0.08 a	0.45±0.04 ab	28.8±1.6 b	0.75±0.01 b	0.18±0.01 a	1.85±0.07 a	0.49±0.03 b	32.6±1.8 b
ANOVA	C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	T	**	*	ns	*	*	**	**	**	**	**
	C x T	ns	ns	**	*	ns	ns	ns	ns	ns	ns

Within each column and for each cultivar, means superscript with different letters are significantly different (ANOVA analysis: ** -P< 0.0 1, * - P <0.05 and ns- none significant). Degrees of freedom for cultivars (C), treatments (T) and interaction effects (C×T) are; 2, 2 and 4, respectively.

Table 4.4: Mean values and ANOVA results of Chl a (µg/g FW), Chl b (µg/g FW), Car (µg/g FW) and proline (µmol g^{-1} FW) in different osmotic stress treatments after a 14 d-period of osmotic stress (T, MPa) and a 2 week-period of recovery for pistachio cultivars (C). Values are means ± SE of nine replicates.

		DROUGHT				RECOVERY			
C	T	Chl *a*	Chl *b*	Car	Proline	Chl *a*	Chl *b*	Car	Proline
Akbari	-0.1	933.0±65.7 a	355.5±20.1 a	269.7±16.9 a	3.7±0.2 b	1048.5±35.9 a	348.0±11.9 a	320.6±13.2 a	3.5±0.6 a
	-0.75	781.2±59.6 ab	320.5±10.6 ab	266.2±16.4 a	6.8±2.1 b	753.1±62.5 b	302.1±16.9 a	259.4±18.6 b	6.4±2.2 a
	-1.5	618.7±97.3 b	254.5±37.6 b	211.0±30.9 a	24.8±2.0 a	601.2±54.3 b	289.8±34.4 a	233.7±10.8 b	11.9±4.6 a
Kaleghochi	-0.1	992.9±78.9 a	394.6±35.1 a	293.7±22.8 a	5.7±1.4 a	1091.0±65.1 a	355.8±20.7 a	336.8±24.5 a	4.8±1.1 b
	-0.75	742.2±66.7 b	321.1±31.5 ab	247.5±17.0 a	14.4±5.5 a	772.6±40.2 b	288.4±14.3 ab	264.5±10.4 b	7.5±2.0 b
	-1.5	612.4±89.2 b	247.7±34.7 b	209.2±30.6 a	13.8±5.1 a	727.9±74.9 b	285.1±25.1 b	247.0±18.9 b	19.7±6.4 a
Ohadi	-0.1	903.1±95.4 a	380.1±36.5 a	257.1±24.8 a	5.0±0.9 a	1106.7±71.5 a	360.2±27.1 a	323.8±17.6 a	3.7±1.0 a
	-0.75	729.1±58.3 a	299.7±18.2 ab	243.8±13.2 a	9.6±2.6 a	705.6±36.4 b	274.1±12.8 b	253.0±11.0 b	6.6±1.2 a
	-1.5	756.0±31.7 a	282.5±12.9 b	259.8±8.7 a	5.4±1.0 a	805.7±35.9 b	289.4±12.0 b	280.9±10.6 ab	5.2±0.8 a
ANOVA	C	ns	ns	ns	ns	ns	ns	ns	*
	T	**	**	ns	**	**	**	**	**
	C x T	ns	ns	ns	**	ns	ns	ns	*

Within each column in every cultivar, means superscript with different letters are significantly different (ANOVA analysis: ** - $P< 0.0\ 1$, * - $P<0.01$ and ns- none significant). Degrees of freedom for cultivars (C), treatments (T) and interaction effects (C×T) are; 2, 2 and 4, respectively.

4.3.6 Proline

Proline accumulation was observed with increasing osmotic stress. Although no significant differences between cultivars occurred, proline content was significantly higher for Akbari and levels tended to be higher for Kaleghochi. Two weeks of recovery were not sufficient for proline to fall back to the control level, an overall significant increase for -1.5 MPa was still found, as well as significant interaction between treatment and cultivar (Table 4.4).

4.3.7 Biomass characterization

Drought stress significantly decreased plant fresh weight (PFW), plant dry weight (Figure 4.9) and leaf dry weight for Akbari and Ohadi, while a decreasing trend was found for Kaleghochi. Although root dry weight was not affected by the osmotic stress treatments, root/shoot ratio increased in all cultivars but this was significant in Kaleghochi (Table 4.5). Control plants showed stem elongation growth during the experimental period, but a growth arrest was noted for all three cultivars under drought stress (Table 4.5). Plant diameter was not affected by treatments (data not shown) although Kaleghochi plants had a significantly higher stem diameter than Akbari and Ohadi, respectively 0.7 mm compared to 0.5 mm and 0.4 mm ($P< 0.05$).

Decreasing osmotic potential of the nutrient solution significantly decreased leaf nitrogen content (Table 4.5), whereas effects of cultivar and interaction were not significant. A correlation study showed that *Pn* positively correlated with leaf nitrogen content during both drought and recovery (Table 4.6 and Figure 4.10). There were also positive significant correlations between leaf nitrogen content and *gs*, C_i, E, $F_v/F_{m,}$ Chl *a*, and stem elongation during both drought and recovery in the three pistachio cultivars (Table 4.6).

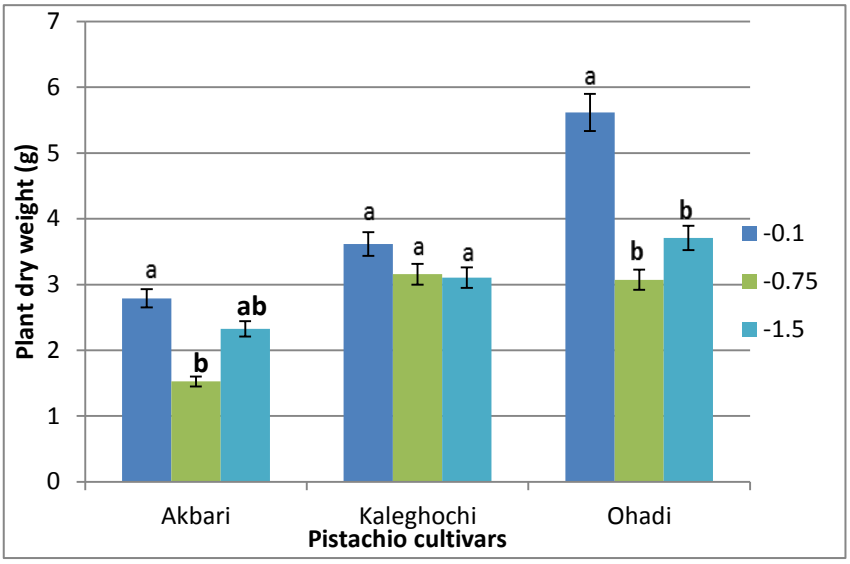


Figure 4.9: Changes in plant dry weight in three pistachio cultivars (i.e. Akbari, Kaleghochi and Ohadi) at control (-0.1 MPa), and different drought stress levels (-0.75 and -1.5 MPa) induced by PEG (n = 9). Within each cultivar, means superscript with unlike letters are significantly different ($P< 0.05$).

Table 4.5: Effects of a 14 d-period of osmotic stress (T, MPa) followed by a 2-week recovery period on plant fresh weight (PFW, g), leaf dry weight (LDW, g), root dry weight (RDW, g), root/shoot ratio (RDW/SDW), stem elongation (SEL, cm) and leaf nitrogen (N, % DW) of pistachio cultivars (C). Values are mean ± SE of nine replicates for plant growth parameters and three replicates for nitrogen content.

C	T	PFW	LDW	RDW	RDW/SDW	SEL	N
Akbari	-0.1	9.3±1.2 a	0.8±0.2 a	1.0±0.2 a	0.58±0.07 a	4.9±1.3 a	2.42±0.10 a
	-0.75	4.3±0.3 b	0.2±0.1 b	0.7±0.1 a	0.86±0.10 a	0.3±0.1 b	1.33±0.04 b
	-1.5	5.9±0.9 b	0.4±0.1 b	1.0±0.2 a	0.80±0.12 a	0.14±0.1 b	1.46±0.08 b
Kaleghochi	-0.1	11.9±3.5 a	1.2±0.4 a	1.2±0.4 a	0.51±0.03 b	8.6±3.0 a	2.47±0.16 a
	-0.75	8.4±1.4 a	0.5±0.2 a	1.5±0.3 a	0.87±0.08 a	0.4±0.2 b	1.44±0.02 b
	-1.5	7.5±1.6 a	0.7±0.2 a	1.2±0.3 a	0.65±0.07 ab	0.7±0.3 b	1.40±0.05 b
Ohadi	-0.1	15.2±2.9 a	1.6±0.4 a	1.7±0.4 a	0.54±0.07 a	11.7±2.9 a	2.47±0.03 a
	-0.75	7.3±0.9 b	0.5±0.2 b	1.2±0.2 a	0.71±0.08 a	0.5±0.16 b	1.54±0.06 b
	-1.5	8.8±1.2 ab	0.6±0.1 b	1.5±0.2 a	0.68±0.06 a	0.12±0.07 b	1.36±0.03 c
ANOVA	C	ns	ns	*	ns	ns	ns
	T	**	**	ns	**	**	**
	C x T	ns	ns	ns	ns	ns	ns

Within each column in every cultivar, means superscript with different letters are significantly different (ANOVA analysis: ** - $P< 0.0\ 1$, * - $P<0.01$ and ns- none significant). Degrees of freedom for cultivars (C), treatments (T) and interaction effects (C×T) are; 2, 2 and 4, respectively.

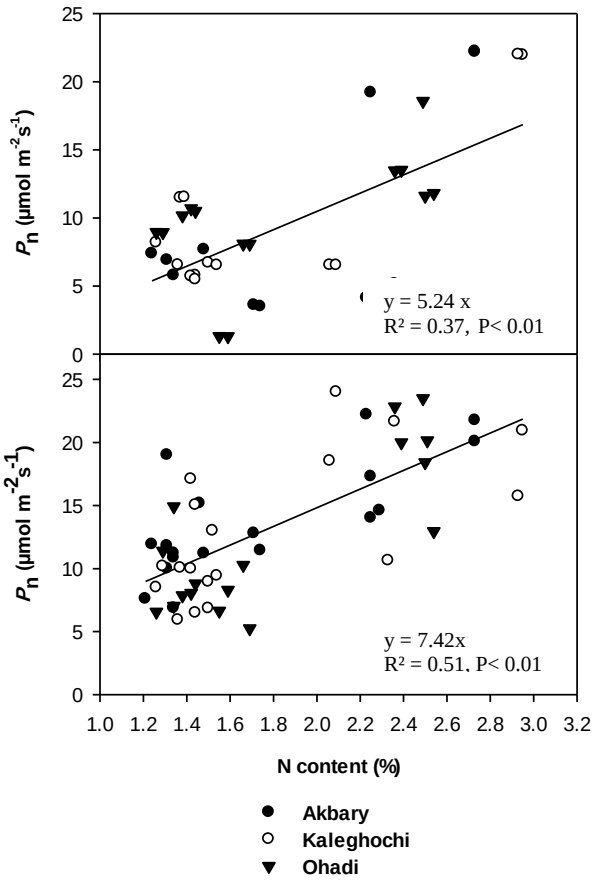


Figure 4.10: Relationship between leaf nitrogen content (%) and *Pn* for the three pistachio cultivars exposed to a 14 d-period of osmotic stress (top) and a 2 week-period of recovery (bottom). Each symbol represents a value for every cultivar as given in Table 4.2; the line represents the following linear relationship: y= 5.24x; R^2= 0.37; n=27; $P < 0.01$(Drought stage) and y= 7.42x; R^2= 0.51; n=27; $P < 0.01$(Recovery stage).

Table 4.6: Overall correlation coefficient analysis of *Pn*, *gs*, C_i, E, F_v/F_m, Chl a, SEL and N after a 14 d-period of osmotic stress (T, MPa) and after a 2 week-period of recovery for pistachio cultivars.

					DROUGHT			
	Pn	*gs*	C_i	E	F_v/F_m	Chl a	SEL	N
Pn	1							
gs	0.97**	1						
C_i	0.64**	0.73**	1					
E	0.97**	0.99**	0.76**	1				
F_v/F_m	0.38**	0.41**	0.43**	0.42**	1			
Chl a	0.30*	0.38**	0.47**	0.39**	0.29*	1		
SEL	0.31*	0.36**	0.42**	0.38**	0.39**	0.21 ns	1	
N	0.63**	0.72**	0.58**	0.70**	0.34*	0.40**	0.61**	1
					RECOVERY			
Pn	1							
gs	0.87**	1						
C_i	0.63**	0.74**	1					
E	0.94**	0.96**	0.78**	1				
F_v/F_m	0.38 ns	0.16 ns	-0.02 ns	0.23 ns	1			
Chl a	0.62**	0.54**	0.33 ns	0.58**	0.47*	1		
SEL	0.52**	0.48*	0.37 ns	0.47*	0.43*	0.66**	1	
N	0.74**	0.65**	0.52*	0.72**	0.63**	0.58*	0.59*	1

4.4 Discussion

Midday leaf water potential (Ψ_l) is a sensitive indicator of drought stress (Naor, 1999). Decreasing leaf water potential with increasing drought stress is known as a mechanism of plants to survive drought stress conditions (Anjum et al., 2011; Ashraf et al., 2004; Rouhi et al., 2007). Drought-tolerant plants are expected to reach lower leaf osmotic potentials than drought-sensitive ones when exposed to water deficit conditions (Anjum et al., 2011; Porcel and Ruiz-Lozano, 2004; Rouhi et al., 2007). In this study, midday leaf water potential became more negative with increasing osmolality of the nutrient solution. Under severe osmotic stress Ψ_l reached -1.05, -1.10 and -1.13 MPa for Akbari, Kaleghochi and Ohadi, respectively (Figure 4.7) indicating that osmotic adjustment is present in the three cultivars. Yet, the decrease compared with the control ($\Delta\Psi_l$) was limited, and averaged 0.29-0.30 MPa for the three cultivars. This reflects the isohydric behavior of the cultivars as already shown by Fardooei (2001) for *P. khinjuk* and *P. mutica.* Tardieu and Simonneau (1998) classified plant species as isohydric if midday Ψ_l remains similar across varying soil water deficits and a tight stomatal control is present. Stomatal closure protected against water loss and resulted in a higher intrinsic WUE under drought stress (Table 4.2); though no cultivar differences were found. A recovery of two weeks reduced $\Delta\Psi_l$ to 0.09-0.14 MPa suggesting that some osmotic adjustment was still operational.

Net photosynthesis under well-watered conditions was higher in leaves of Akbari and Ohadi than that of Kaleghochi. Furthermore, photosynthetic rates in this study (Table 4.2) were higher than those found for *P. khinjuk* and *P. mutica* which ranged from 9.7 to 11.7 µmol CO_2 $m^{-2}s^{-1}$, respectively (Ranjbarfordoei et al., 2000). Exposure to a 14-d drought stress period significantly declined photosynthesis and *gs* for the three studied pistachio cultivars, although photosynthetic rates remained higher in Ohadi than in the two other cultivars under severe drought. These results are in agreement with previous work on *Pistachio* species (Bagheri et al., 2011; Behboudian et al., 1986; Germana, 1996; Goldhamer et al., 1985). A recovery phase of 14 days, however, was insufficient for restoring the plant's photosynthetic capacity. The *gs* of these plants remained at a lower level than that of well-watered control plants after 2 weeks of recovery (Table 4.2) (Flexas et al., 2009; Gallé and Feller, 2007). This indicates that the photosynthetic performance of pistachio cultivars did not recover fast from the applied osmotic stress. Romero *et al.* (2004a) found a range of responses in drought-stressed almond (*Prunus dulcis*) varieties, going from partial recovery to rapid recovery of

photosynthetic capacity clearly showing varietal differences. In general, plants submitted to mild drought stress recover fast, after within one day (Chaves *et al.*, 2009), whereas plants submitted to severe drought stress regain only 40-60 % of their maximum photosynthesis ability during the day after re-watering, and recovery continues during the following days. Sometimes maximum photosynthesis rates are not recovered even after a number of days (Chaves et al., 2009), which was also the case for all three pistachio cultivars in our experiment.

Both stomatal and non-stomatal limitations can reduce net photosynthesis (Behboudian et al., 1986; Fardooei, 2001; Flexas et al., 2009). In our test, the reductions in *Pn* and *gs* were accompanied by a reduction in C_i (Table 4.2). The cultivars responded very similarly with a strong reduction in C_i at Ψ_s= -0.75 MPa. This decrease in C_i indicates that stomatal limitations dominate with moderate osmotic drought stress, irrespective of any metabolic impairment. This can also be concluded from the relation between C_i/C_a and *gs* (Figure 4.8) as no inflection point [where the ratio increases again at low *gs*] were found. In most cases, the point at which C_i starts to increase occurs at a *gs* value of around 0.050 mol H_2O m^{-2} s^{-1} (Flexas et al., 2002). Such low *gs* values were hardly reached in our experiment; thus, stomatal conductance could be the dominating factor limiting photosynthesis in the studied pistachio cultivars.

Yet, non-stomatal factors can also affect photosynthesis. Ogren and Oquist (1985) reported that the degree of drought stress defines how *Pn* is affected: under low drought stress, *Pn* is limited by stomatal closure, whereas under more severe drought stress non-stomatal factors prevail. Slow recovery of photosynthesis in our pistachio cultivars might indicate that oxidative stress occurred. Overproduction of ROS in plant cells under stress can damage cellular components, including DNA, proteins and membrane lipids (Gill and Tuteja, 2010; Rejeb et al., 2014), and lead to destruction of photosynthetic pigments (Yordanov et al., 2000).

Moreover, *gs* remained relatively low with values between 0.10 and 0.20 mol H_2O m^{-2} s^{-1} after 2 weeks of recovery. Indeed, the decline in F_v/F_m for Akbari both under drought treatment and after recovery indicates damage to PSII (Maxwell and Johnson, 2000). We also found significant correlations between photosynthetic rates and F_v/F_m during drought stress ($P< 0.01$). Similar results were found with *P. vera* Ahmadaghaii under drought stress (Habibi and Hajiboland, 2013), although F_v/F_m was not affected for *P.vera* Badami and Qazvini (Bagheri et al., 2011). In our study, the down-regulation of YII and qP was more apparent

during the recovery phase. Thermal dissipation as indicated by higher NPQ values led to removal of excess excitation energy at this point for all three cultivars. Ranjbarfordoei et al. (2000) also found that thermal dissipation increased under drought stress in *P. mutica* and *P. khinjuk.* This excess excited energy favors high production rates of ROS and might be partly responsible for the degradation of chlorophyll pigments in the three cultivars. Photosynthetic pigments play a major role in maintaining photosynthetic ability of most plants. There were significant correlations between photosynthetic rates with Chl *a* contents in both drought ($P< 0.05$) and recovery ($P< 0.01$) stages. Farghali (1998) linked the decrease in chlorophyll pigments under drought stress not only to degradation, but also to an increase in soluble stress proteins which in turn led to decreased chlorophyll content due to inhibition of proto-chlorophyll synthesis. This decrease in photosynthetic pigments was also observed for cultivated and wild pistachio species under drought conditions (Abbaspour et al., 2012b; Bagheri et al., 2011; Ranjbarfordoei et al., 2000). In the present research, leaf nitrogen content was significantly lower under osmotic stress (Table 4.5) and this reduction correlated well with reduced *Pn* and Chl *a* content during drought and recovery (Table 4.6, Figure 4.10). Photosynthetic rate is generally closely correlated to leaf nitrogen. This close correlation has been attributed to the large fraction of total organic nitrogen allocated to chloroplasts (Evans, 1989). Decreasing photosynthesis under drought stress will in turn impair primary nitrogen assimilation (Bauer et al., 1997; Xu and Zhou, 2006). Lower leaf nitrogen levels are probably also linked to chloroplast degradation under increasing stress as also chlorophyll content decreased (Table 4.4). Reduced leaf nitrogen levels might thus be an early signal for the onset of gradual leaf abscission under drought. Indeed, gradual leaf abscission is an adaptation to drought stress in many plants and Del Arco *et al.* (1991) calculated a N retranslocation percentage averaging 45.6 % for deciduous trees prior to leaf abscission. During the initial but still reversible phase of leaf senescence stromal enzymes are degraded which also leads to the observed photosynthesis decline. Amino acids derived from this enzyme degradation can be exported via the phloem and are translocated to the root system in woody plants (Hörtensteiner and Feller, 2002). In other plant species, such as tomato, remobilization of nitrogen under drought stress occurs to meet the requirements of rapidly developing fruits. The leaf nitrogen pool was 1.5 times higher under drought stress than in control plants, and this was mainly due to an increase of glutamate and aspartate (Bauer et al., 1997).

Under osmotic stress, proline is synthesized mainly from glutamate (Rejeb et al., 2014). Increased accumulation of proline has been correlated with improved tolerance to various

abiotic stresses especially salt and drought (Rejeb et al., 2014; Signorelli *et al.*, 2013). Proline accumulation in plants acts as an osmotic adjustment mediator as well as a component of the non-enzymatic antioxidative defense system (Rejeb et al., 2014; Saradhi, 1991). In this study, proline concentration increased with increasing osmotic stress. Akbari showed even a 6-fold increase for the highest osmotic stress, whereas proline accumulation did not play any significant role in Ohadi and Kaleghochi (Table 4.4). We observed a reduced electron transport rate (ETR) in pistachio under increasing osmotic stress, which would indicate a reduced redox potential regulation (Table 4.3). Proline metabolism is linked to NAD (P) H/NAD $(P)^+$ redox balance (Miller et al., 2010; Rejeb et al., 2014). Increased proline in biosynthesis in chloroplasts during stress can maintain a low NADPH:$NADP^+$ ratio, contributes to sustain the electron flow between photosynthetic excitation centers (ETR), stabilizes the redox balance, and reduces photoinhibition and damage to the photosynthetic apparatus (Hare and Cress, 1997; Rejeb et al., 2014).

Free proline is also an osmoprotectant and will under decreasing leaf water potential-accumulate in the cytosol although vacuolar accumulation of carbohydrates will also contribute. Proline will thus also contribute to the osmotic adjustment of pistachio in our case, especially in Akbari, and will help to maintain leaf turgor pressure. These results are consistent with the results of Panahi (2009) and Ranjbarfordoei et al. (2000) on cultivated and wild pistachio species and Rouhi et al. (2007) on almond species. However, it must be noted that although proline accumulation has been shown to occur in conjunction with osmotic adjustment in drought-tolerant species, it may also be accumulated in drought-susceptible cultivars as a symptom of stress as in cassava (*Manihot esculenta* crantz) (Sundaresan and Sudhakaran, 1996) and *Phaseolus* spp. (Andrade *et al.*, 1995). A higher proline accumulation is not always indicative for a greater drought tolerance (Sánchez-Rodríguez et al., 2010). For instance, a significant rise in proline content was reported under moderate drought stress in cherry tomato, though the lowest level of proline was found in 'Zarin' a drought tolerant cultivar. As for the other studied parameters, a recovery period of two weeks was insufficient to return to basal proline levels for the highest osmotic stress.

Osmotic stress resulted in growth arrest for all cultivars, and no regrowth was observed after 2 weeks of recovery. Under severe water deficiency, cell elongation of higher plants will be inhibited by an interruption in water flow from the xylem to the surrounding elongating cells (Nonami, 1998), but nitrogen content also has noticeable effects on shoot growth of pistachio seedlings (Afrousheh et al., 2010). Thus, in this experiment drought induced

nitrogen reallocation could be another factor that induced growth arrest. Overall growth reduction observed in our experiment can be attributed to a decrease in leaf biomass formation, not to shoot or root biomass production. Decrease in total fresh weight of pistachio cultivars tested with increasing drought is in line with those of other pistachio species (Abbaspour et al., 2012b; Ranjbarfordoei et al., 2000).

Root growth is generally less sensitive to drought stress compared to other biomass components (Hsiao and Xu, 2000). The capacity of osmotic adjustment and turgor maintenance in roots may influence root (root partitioning patterns and root growth) and leaf responses to water deficits through indirect effects of root-produced plant growth (Ranney *et al.*, 1991; Turner, 1986b). An increase in root/shoot ratio has been proposed as one of the mechanisms involved in the adaptation of plants to drought stress (Turner, 1997). In our study, increasing root/shoot ratios under osmotic stress are only related to a decrease in aboveground biomass and more specifically, leaf biomass (Table 4.5).

Under control conditions, Ohadi maintained the highest leaf and root dry weight compared to both other cultivars (Table 4.5) but this cultivar showed the largest decrease for all plant growth parameters in reaction to drought stress. Despite this strong decrease in biomass, the photosynthetic rate of Ohadi plants remained significantly higher under severe osmotic stress compared to the other two cultivars. For plants that grow mainly in semi-arid areas, such as pistachio, a well-developed root system is effective in exploiting deep soil water (Ferguson et al., 2005; Panahi et al., 2002). Ohadi had a higher root mass both in control and osmotic stress treatments, which suggests a better adaptation to semi-arid environments.

4.5 Conclusions

Pistachio trees are considered to be drought-tolerant, yet the applied osmotic drought stress induced significant changes in gas exchange parameters (decreased *Pn* accompanied by decreased *gs*, C_i, and E), reductions in chlorophyll fluorescence parameters (F_v/F_m and qP), reduction in chlorophyll pigments and nitrogen content of leaves and an increase in proline levels. The recovery period from the applied stress was longer than expected as after two weeks of recovery, negative effects of the stress were still present in photosynthesis and chlorophyll fluorescence parameters. Although, MANOVA analysis (Table 4.1) indicated that the results were not significantly different for the three tested cultivars, while interaction

effects between cultivars and treatment was significant. Therefore, there is a possibility of differences between different cultivars and treatments in some measured variables.

Based on the reduction in photosynthesis and increase in proline content, Akbari seems more sensitive to the applied drought stress than Ohadi and Kaleghochi cultivars. However, some caution must be exercised as the results were obtained using PEG and further research in field conditions is needed to confirm this survey's research**.**

Chapter 5. **Impact of osmotic drought stress on the biochemical characteristics, water relations, and nitrogen and carbon isotope composition of pistachio (*Pistacia vera* L.) cultivars**

Adapted from:
Esmaeilpour, A., M. C. Van Labeke, R. Samson and P. Van Damme. 2014. Variation of Relative Water Content, Water Use Efficiency and Stomatal Density during Drought Stress and Subsequent Recovery in Pistachio Cultivars (*Pistacia vera* L.). The 29th international horticultural congress (IHC2014). Brisbane, Australia.

Esmaeilpour, Ali; Van Labeke, Marie-Christine; Samson, Roeland; P. Boeckx; Van Damme, Patrick. 2016. Variation in biochemical characteristics, water status, stomata features, leaf carbon isotope composition and its relationship to water use efficiency in pistachio (*Pistacia vera* L.) cultivars under drought stress condition. Scientia Horticulturae 211, 158-166.

5.1 Introduction

Pistachio is a subtropical fruit and has become one of the dominant agricultural crops in Iran, especially in Kerman province. It is mainly produced in a variety of infertile, salty and alkaline soils and in environments subjected to varying periods of drought stress (Bagheri *et al.*, 2012; Panahi et al., 2002; Sheibani, 1995). Increased establishment of irrigated pistachio orchards during the last decades in this region has decreased the availability of underground water resources and prolonged drought periods is the major concern for the pistachio producers (Esmaeilpour et al., 2010). Competition for the limited water supply available for irrigation of pistachio orchards is increasing. Although pistachio nut trees are drought tolerant, it does not mean that pistachio trees require less water for optimal performance (Esmaeilpour et al., 2010; Sheibani, 1995).

Drought is probably the most important factor limiting crop productivity world-wide (Anjum et al., 2011; Chaves et al., 2002; Praba et al., 2009). Water stress greatly suppresses cell expansion and cell growth as a result of the low turgor pressure (Shao et al., 2008). Turgor pressure maintenance allows stomata to remain open, photosynthesis to continue and growth to be uninterrupted (Anyia and Herzog, 2004; Arndt et al., 2001; Pessarakli, 1994). Stomatal characteristics, such as frequency and sizes, are greatly affected by the species but also by environmental factors (Dong and Zhang, 2000; Munir et al., 2011). In pistachio rootstocks, the highest stomatal density and the lowest stomatal length and width were obtained under severe drought stress (Arzani et al., 2013) and this adaptation allows a better control of the leaf water content. Another common mechanism to maintain the turgor at low water availability is osmotic adjustment. Drought stress has been shown to promote the accumulation of soluble sugars and proline in both the leaves and roots of mycorrhizal and non-mycorrhizal pistachio plants, compared to those under well-watered conditions (Abbaspour et al., 2012b).

Water use efficiency (WUE) is widely used to evaluate the plant adaptation to limited water supply (Araus et al., 2002; Moghaddam *et al.*, 2013). WUE may be estimated as the ratio between net photosynthesis (*Pn*) and transpiration (E), which is known as instantaneous water use efficiency (physiological index) (Mediavilla et al., 2002; Polley, 2002), as the ratio between *Pn* and stomatal conductance (*gs*), which is known as intrinsic water use efficiency (physiological index) (Boyer, 1996; Pascual et al., 2013) and as the ratio of dry matter accumulation over time to the amount of water transpired which is identified as biomass/yield water use efficiency (agronomic index). Physiological WUE indices are widely used in

comparative studies involving plant responses to changes in water supply and demand (Cabrera-Bosquet *et al.*, 2009; Condon et al., 2004) and provide information about the physiological performance in response to short-term changes in the plant water status (Centritto *et al.*, 2002). In pistachio, WUE varies with different fruit growth stages and cultivars (Sajjadinia et al., 2010). It was shown that intrinsic WUE values increased in wild almonds (*Prunus dulcis*) (Rouhi et al., 2007) and *Ziziphus rotundifolia* Lamk. (Rhamnaceae) (Arndt et al., 2001) by increasing drought stress. In contrast, WUE was not affected under drought in two wild pistachio species (*P. mutica* and *P. khinjuk*) (Ranjbarfordoei et al., 2002).

A decreasing stomatal conductance results in increasing WUE and declining leaf intercellular CO_2 (*Ci*) and consequently decreasing carbon isotope discrimination (Δ). Therefore, there should be a negative relation between WUE and Δ due to the independent relation between *Ci* and Δ or WUE (Farquhar et al., 1989; Farquhar and Sharkey, 1982; Saugier *et al.*, 2012). In breeding programs, the variation in the CO_2 assimilation to stomatal conductance or water transpiration ratio can be exploited by indirect selection for WUE via Δ (Moghaddam et al., 2013). Carbon isotope discrimination (Δ) decreased with increasing salinity in the leaves, stems and roots of pistachio (*P. vera*) seedlings (Hokmabadi et al., 2005). Yet, carbon isotope discrimination values can vary in different plant species. Alternatively the leaf carbon isotope composition ($\delta^{13}C$) can be measured. The latter parameter is positively linked to instantaneous WUE and intrinsic WUE in C_3 plants (Farquhar et al., 1989) especially in wheat (Cabrera-Bosquet et al., 2009), and grapevine (de Souza *et al.*, 2005). Carbon isotope ratios of *Pistacia lentiscus*, *Quercus ilex* and *Phillyrea argustifoli* showed similar $\delta^{13}C$ values, while two deciduous oak species, *Q. pubescens* and *Q. cerris*, had lower $\delta^{13}C$ values in a Mediterranean ecosystem (Valentini et al., 1992).

Decreasing water availability will also reduce the N availability and use and this could be assessed by nitrogen isotope composition ($\delta^{15}N$). In durum wheat (*Triticum durum*) root $\delta^{15}N$ increased under drought stress while the same stress treatments significantly decreased shoot $\delta^{15}N$ (Yousfi et al., 2012). In broccoli (*Brassica oleracea*), it was found that high salinity significantly decreased nitrogen isotope discrimination and increased carbon isotope discrimination in leaf dry matter (Del Amor and Cuadra-Crespo, 2011). Carbon and nitrogen isotope ratios constitute a tool that is widely used to detect environmental effects especially in forests and herbaceous plants and to link these effects to water stress, WUE and N availability (Pascual et al., 2013). However, to our knowledge, uses of C and N isotope ratios to assess drought stress in fruit trees are very limited.

There is a wide genetic variation of edible pistachio (*P. vera*) cultivars in Iran (Esmaeilpour et al., 2010; Esmaeilpour and Khezri, 2006; Sheibani, 1995). They have originated from natural pistachio forests in the northeast part of the country and are grown in different environmental conditions. Although pistachio is well-known for its drought tolerance (Abbaspour et al., 2012b; Bagheri et al., 2011; Esmaeilpour et al., 2015; Fardooei, 2001; Habibi and Hajiboland, 2013; Khoyerdi et al., 2016; Panahi, 2009; Tajabadipour et al., 2006), limited evidence is available on the underlying mechanisms of drought tolerance associated with the plant's water relations, and especially carbon and nitrogen isotope composition.

The objectives of this investigation were: (1) to evaluate the effects of osmotic drought stress on RWC, carbohydrate content and stomatal characteristics of three pistachio cultivars; (2) to determine the effect of drought stress on WUE, $\delta^{13}C$ and $\delta^{15}N$ under drought stress conditions; (3) to study the relationship between WUE and $\delta^{13}C$; (4) to assess the use of carbone isotope composition in pistachio trees as an indicator of water use efficiency (WUE) under drought stress.

5.2 Materials and methods

5.2.1 Plant material and experimental set-up

Plants and experimental conditions are similar to those presented in 4.2.1

5.2.2 Measurements

5.2.2.1 Carbohydrate analysis

Pistachio leaf samples were taken from the top of each plant at the end of the drought stress and the recovery, respectively. Leaf samples were kept in an icebox to minimize evapotranspiration and were immediately transferred to the laboratory. Then leaves were grinded with liquid nitrogen (N_2) (Figure 5.1, A & B) and kept at -80 ºC until analysis (Figure 5.1, C). Sugars were extracted from 300 mg of fresh leaf material with 80% ethanol following Van Labeke and Degeyter (2004). Soluble carbohydrates (glucose, fructose and sucrose) contents were analysed (nine replicates) by high pH anion-exchange chromatography with pulsed amperometric detection (Waters; column CarboPac PA100 with companion guard column, eluent: 50 mM NaOH, temperature: 22 ºC). The remaining

ethanol-insoluble material was washed twice with ethanol 80 % and the residual pellet was treated with HCl 1 M for 2 h at 95 ºC for starch hydrolysis. Starch concentrations were determined by spectrophotometer at 340 nm by the enzymatic reduction of NADP+ (Uvikon XL, Bio-Tek Instrument, Winooski, USA).

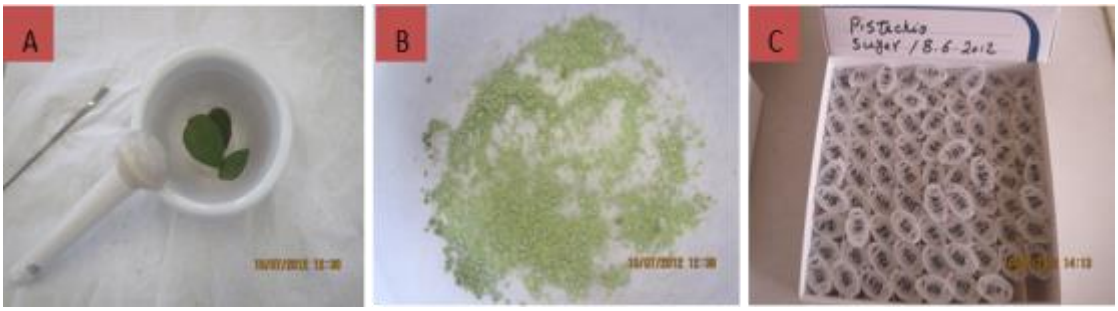


Figure 5.1: Pulverization of pistachio leaves with liquid nitrogen (A and B) and keeping them at -80 ºC (C).

5.2.2.2 Relative Water Content (RWC)

RWC was determined on fully expanded leaves (nine replicates) according to the method of Merino *et al.* (1976). From each plant, one leaf at the end of the second drought stress and recovery week was sampled and immediately transferred to the laboratory in an icebox. There we determined fresh leaf weight (FW) by digital balance (Mettler Toledo PB602-L, Greifensee, Switzerland). In order to obtain turgid weight (TW), leaf disks were floated in distilled water inside a closed Petri dish and kept in a fridge for 24 h. Subsequently, the water was gently wiped from the leaf surface with tissue paper and the turgid weight measured. Afterward, leaf samples were placed in an electric oven at 85 ºC for 48 h in order to obtain leaf dry weight (DW). Values of FW, TW, and DW were then used to calculate RWC, using the following equation: RWC (%) = [(FW - DW)/ (TW - DW)] × 100 %, where FW, DW and TW are fresh, dry and turgid weight (g), respectively.

5.2.2.3 Water Use Efficiency (WUE)

Gas exchange measurements were done in this experiment as already described in 4.3.3. Following Ashraf (2002) and Mediavilla et al. (2002), *Pn* /E ratio was taken as an estimate of instantaneous water use efficiency (WUE), and the ratio between *Pn*/*gs*, which is known as intrinsic water use efficiency (Boyer, 1996; Pascual et al., 2013).

5.2.2.4 Stomatal characteristics

Fully expanded leaves from each treatment and cultivar were used for stomatal density measurement at the abaxial leaf side using the replica method (Rouhi, 2007). Replicas were analysed at a magnification of 400x using a bright field microscope (Olympus CX41, Olympus Corporation, Tokyo, Japan). To facilitate stomata counting, the image from the microscope was transferred to a TV screen (Figure 5.2, A) by means of a video camera (JVC TK - 860 E). The number of stomata was directly counted on the TV screen and converted to stomatal density (number of stomatal per mm^2). For each treatment, stomata of 81 fields (samples) were counted (9 replications in each treatment x 3 imprint areas per replication x 3 counts per area) according to the method of Zaid and Hughes (1995). Stomatal cell length and width (Figure 5.2, B) were measured on 12 stomata per plant directly in the snapshot of the screen. As there was no new growth of the drought exposed plants, stomata on leaf samples from those plants were not formed after stress.

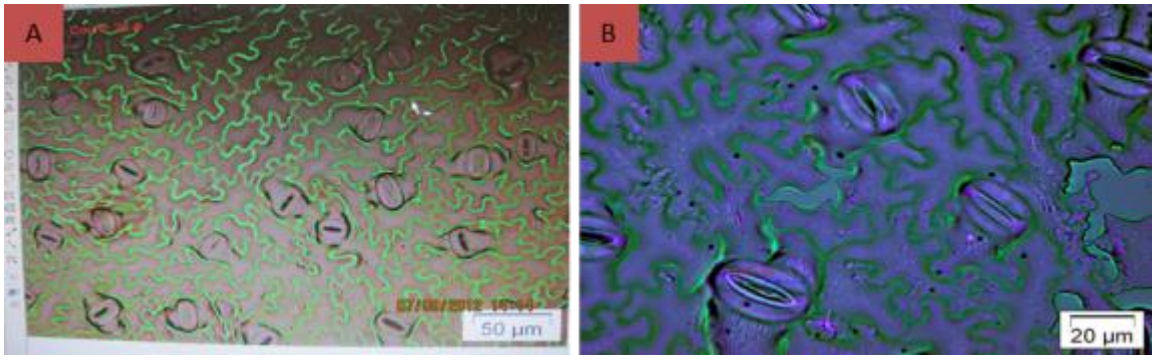


Figure 5.2: Counting of pistachio leaf stomata (A) and measurement of stomatal length and width (B).

5.2.2.5 Carbon and nitrogen isotope analyses

Five mg subsamples of ground dry plant material (ZM200, Retsch, Germany) were packed (three replicates per treatment and rootstock) in tin capsules and analysed for ^{13}C presence by combustion to CO_2 in the presence of O_2 by an elemental analyser (EA) (ANCA-SL, SerCon, UK) coupled to an isotope ratio mass spectrometer (IRMS) (model 20–20, SerCon, UK) to measure total C and $\delta^{13}C$, and N and $\delta^{15}N$ (Staelens et al., 2012) (Figure 5.3, A- C). Nitrogen and carbon contents were expressed as percentage of dry matter. Carbon isotope composition (δ) was calculated relative to the international Pee Dee Belemnite (PDB) standard (Farquhar

et al., 1989): $\delta^{13}C = [(R_{sa}/R_{sd} -1) \times 1000]$ (‰) where R_{sa} and R_{sd} are the ^{13}C:^{12}C ratios of the sample and standard, respectively. To calculate nitrogen isotope composition the same formula for carbon was used for the ^{15}N: ^{14}N ratio ($\delta^{15}N$) = $[(R_{sa}/R_{sd} -1) \times 1000]$ (‰), where R_{sa} and R_{sd} are the ^{15}N: ^{14}N ratio of the sample and standard, respectively but in this case the standard refers to N_2 in air.

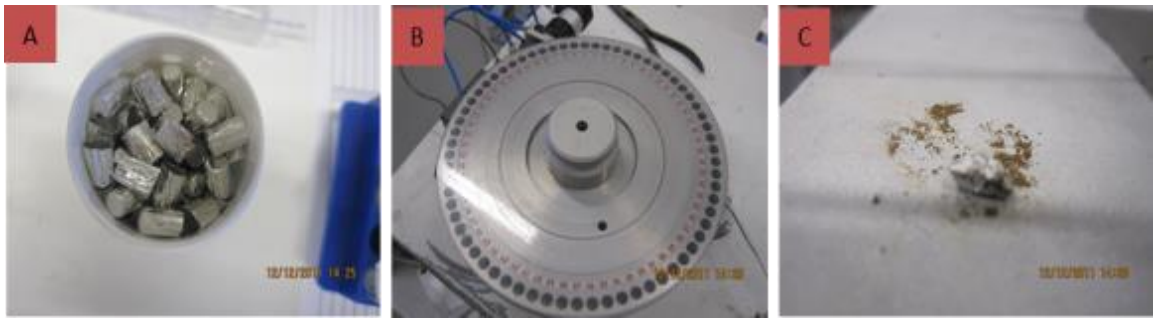


Figure 5.3: Packing of ground leaf plant material in tin metal capsule to analyze subsample for ^{13}C and ^{15}N presence by an elemental analyzer.

5.2.3 Statistical analysis

The experiment was designed as a randomized complete block design (RCBD) with nine treatment levels (three cultivars* three drought stress levels) and nine replications. A two-way analysis of variance was used to test for drought treatment differences and cultivar effects. One-way ANOVA to test treatments by cultivar was also performed. Means were compared by Tukey's test. Correlations between parameters were calculated using Pearson's correlation method. Principal component analysis (PCA) was carried out on all measured parameters in chapter 4 and 5. Only PCA with eigenvalues > 1, thus explaining more than a single parameter alone, were extracted. In this experiment we have 3 cultivars × 3 treatments/water stresses (3 × 3= 9 treatment levels) with 9 replicates and 37 variables/vectors. Therefore, we have an 81 × 37 matrix and run the PCA for this matrix. In the biplot the mean values of the replications were inserted, while all of data were used for PCA and all other statistical analyses. A normality test of data distribution was conducted using descriptive statistical parameters and a homogeneity test was done to see diverse data using Levene's test of equality of error variance by SPSS software. All analyses were performed by SPSS version 20 (IBM Corporation, USA).

5.3 Results

5.3.1 Changes in carbohydrate concentrations

Sucrose content significantly accumulated under severe drought stress in all three cultivars (Table 5.1). Fructose significantly increased in Kaleghochi for the highest drought stress level. Also, starch accumulated in all three cultivars at both drought stress levels (-0.75 and -1.5 MPa). When compared to their corresponding control plants, these changes were significant in Ohadi and non-significant in Akbari and Kaleghochi cultivars. Although the ANOVA results for treatment effects were significant with exception of glucose, there were no significant interaction effects of treatments and cultivars. Also cultivar effects were non-significant with exception for fructose (drought stage) and starch (recovery stage). No significant effect on glucose levels was observed (Table 5.1).

After two recovery weeks, plants that have been highly drought-stressed still had significantly higher amounts of glucose, fructose, sucrose and starch compared to the control for all three cultivars. No significant interaction between cultivar and treatment for these traits were observed (Table 5.1). A correlation analysis showed that there were positive significant correlations ($P< 0.05$) between soluble sugars and intrinsic and instantaneous water use efficiency. This correlation analysis also showed negative significant correlations between soluble sugars and biomass ($P< 0.05$) at recovery stage (Table 5.4).

5.3.2 Relative Water Content (RWC)

RWC was not affected for Akbari and Ohadi under drought stress. However, RWC significantly decreased for Kaleghochi and ranged from 92 % for the control to 85.9 % for the mild and 80.6 % for the severe drought stress, respectively (Figure 5.4, A).

During recovery, RWC values in drought-stressed treatments were significantly lower compared to their respective controls for Kaleghochi and Ohadi cultivars, whereas no differences were found for Akbari (Figure 5.4, B). In general, treatment effects were significant during both drought ($P< 0.05$) and subsequent recovery ($P< 0.01$) phases. No overall cultivar effects were found (data not shown).

5.3.3 Stomatal characteristics

Severe drought stress treatment (-1.5 MPa) significantly decreased stomatal length and width compared to control for Akbari (Table 5.2). Yet, no effects of drought stress treatments were found for stomatal length and width for Kaleghochi and Ohadi. Effects of severe

drought stress treatments on stomatal density were not significant compared to the control treatment in all three cultivars; whereas mild drought stress treatment decreased the stomatal density in the Kaleghochi cultivar (Table 5.2). Akbari had the highest stomata density, whereas Ohadi had the lowest stomatal density in the control treatment (Table 5.2). The effect of cultivar and interaction effects differed significantly for the stomatal density ($P < 0.01$), although treatment effects were not significant. There was a weak negative relationship between stomatal density with stomatal width (R^2= 0.22) and length (R^2= 0.14) (Figure 5.5).

Table 5.1: Mean values of glucose, fructose, sucrose and starch (g/100 fresh weight) at different drought stress levels, in both the drought and recovery stages for three pistachio cultivars. Abbreviations are treatment (T), cultivar (C). Values are means ± SE of nine replicates.

		DROUGHT				RECOVERY			
Cultivars	T (MPa)	glucose	fructose	sucrose	starch	glucose	fructose	sucrose	starch
Akbari	-0.1	0.34±0.06 a	0.39±0.05 a	0.49±0.08 b	1.18 ±0.25 a	0.22±0.03 b	0.23±0.03 b	0.20±0.05 b	0.65 ±0.07 b
	-0.75	0.47 ±0.10 a	0.58±0.12 a	0.73 ±0.09ab	1.33± 0.20 a	0.40±0.07 ab	0.56±0.11 ab	0.57±0.11 a	1.20±0.13 ab
	-1.5	0.42±0.05 a	0.54±0.07 a	0.99 ±0.16a	1.89± 0.26 a	0.54±0.13 a	0.66±0.14 a	0.76±0.09 a	1.90±0.39 a
Kaleghochi	-0.1	0.32± 0.06 a	0.28±0.04 b	0.66±0.19 b	1.43±0.39 a	0.25±0.02 b	0.26±0.02 b	0.25± 0.08 b	0.76±0.09 b
	-0.75	0.24±0.03 a	0.29±0.02 b	0.58±0.05 b	1.44±0.17 a	0.31±0.04 ab	0.44±0.08 ab	0.79±0.13 a	1.95±0.18 a
	-1.5	0.41±0.06 a	0.50±0.08 a	1.03±0.12 a	2.13±0.30 a	0.53±0.13 a	0.70±0.16 a	0.79±0.14 a	1.73±0.39 a
Ohadi	-0.1	0.23±0.02 a	0.25±0.03 a	0.44±0.09 b	0.91±0.17 b	0.25±0.03 a	0.28±0.04 b	0.30±0.07 b	0.90±0.20 b
	-0.75	0.26±0.02 a	0.33±0.03 a	0.65±0.14 ab	1.88±0.25 a	0.27±0.04 a	0.35±0.05 ab	0.55±0.06 ab	2.12±0.25 a
	-1.5	0.38±0.11 a	0.37±0.08 a	0.96± 0.09 a	2.44±0.26 a	0.30±0.04 a	0.44±0.05 a	0.72±0.10 a	2.37±0.25 a
ANOVA	C	ns	**	ns	ns	ns	ns	ns	*
	T	ns	*	**	**	**	**	**	**
	C*T	ns	ns	ns	ns	ns	ns	ns	ns

Within each column in every cultivar, means superscript with different letters are significantly different by Tukey's range test ($P< 0.05$). (* significant at ($P< 0.05$), ** significant at ($P< 0.01$), and ns, not significant).

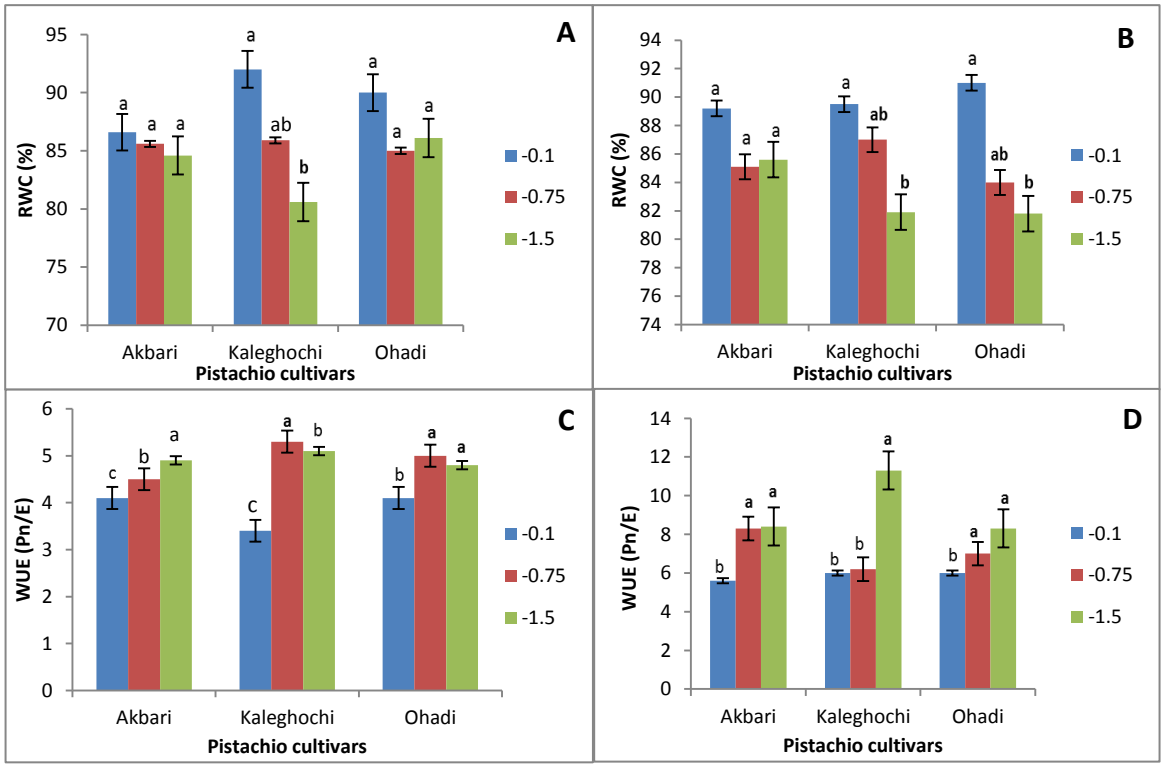


Figure 5.4: Changes in RWC, during drought (A) and recovery stage (B), and instantaneous WUE, in drought (C) and recovery stage (D), of plants exposed to -0.1, -0.75 and -1.5 MPa drought stress levels in three Iranian pistachio cultivars. Data are means of six replications. For each column, different letters indicate significant differences ($P<0.05$), according to Tukey's range test.

Table 5.2: Mean values of stomatal length (SL, µm), stomatal width (SW, µm) and stomatal density (SD) in different drought stress treatments (T) for three pistachio cultivars (C) i.e. Akbari, Kaleghochi and Ohadi. Values are means ± SE of nine replicates.

Cultivars	T (MPa)	SL (µm)	SW (µm)	SD
Akbari	-0.1	28.6±0.56 a	17.4±0.58 a	293.2±9.10 a
	-0.75	27.4±0.76 ab	16.3±0.53 ab	328.7±28.24 a
	-1.5	26.7±0.51 b	16.2±0.26 b	290.2±11.63 a
Kaleghochi	-0.1	27.0± 0.83 a	16.6±0.46 a	274.3±15.05 ab
	-0.75	28.4±0.70 a	17.6±0.53 a	244.1±7.31 b
	-1.5	27.9±0.86 a	17.3±0.47 a	306.2±9.61 a
Ohadi	-0.1	27.7± 0.42 a	17.2± 0.22 a	270.3±8.69 a
	-0.75	28.5±0.63 a	17.3±0.43 a	245.2±13.50 a
	-1.5	28.1±0.39 a	17.7± 0.31 a	247.6±17.78 a
ANOVA	C	ns	ns	**
	T	ns	ns	ns
	C*T	ns	ns	**

Within each column in every cultivar, means superscript with different letters are significantly different by Tukey's range test ($P< 0.05$). (* significant at ($P< 0.05$), ** significant at ($P< 0.01$), and ns not significant).

5.3.4 Intrinsic and instantaneous WUE

Under drought stress both intrinsic WUE (*Pn*/*gs*) and instantaneous WUE (*Pn*/E) increased for all cultivars (Table 5.3). Increased percentages in instantaneous WUE (*Pn*/E) were 50.6 %, 55.1 % and 38.2 % under severe drought stress level compared to their corresponding controls for Akbari, Kaleghochi and Ohadi after two weeks stress, respectively (Figure 5.4, C). After two weeks of recovery there was still a significant difference between plants that had been subjected to the high drought stress compared to their control in all three pistachio cultivars (Figure 5.4, D). At recovery stage, main treatment effects were significant in the severe drought stress treatment compared to corresponding control plants for both intrinsic and instantaneous water use efficiency (Data not shown).

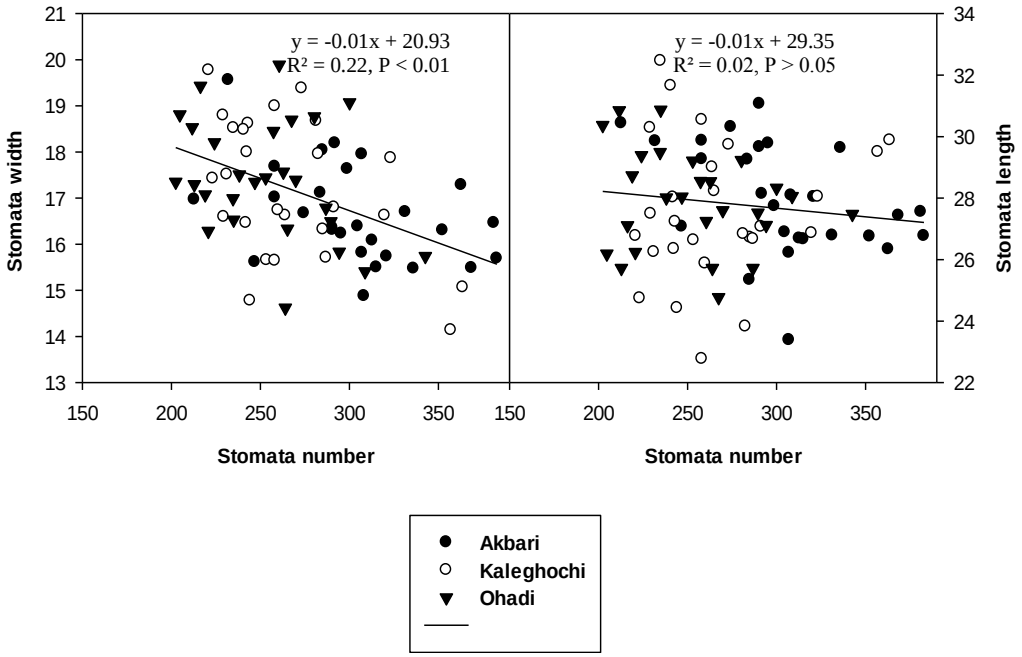


Figure 5.5: Relation between leaf stomata number (density) with stomata width (left) and stomata length (right) in pistachio cultivars exposed to osmotic drought stress. Each symbol represents a mean value for every cultivar as given in Table 5.2; the line represents the following linear relationship: y= -0.01x + 20.93; R^2=0.22; n=27; $P < 0.01$(Stomata width) and y= -0.01x + 29.35; R^2= 0.02; n=27; $P > 0.05$ (Stomata length).

5.3.5 Carbon and nitrogen isotope composition

Carbon isotope composition ($\delta^{13}C$) significantly increased in different drought stress treatments in Akbari cultivar. While the impact of drought stress treatments on $\delta^{13}C$ was not significant compared to corresponding control in Kaleghochi and Ohadi pistachio cultivars (Table 5.3).

Nitrogen isotope composition ($\delta^{15}N$) decreased in both drought stress treatments (-0.75 and -1.5 MPa) compared to control. In addition, the lowest value of nitrogen isotope composition was obtained in the severe (-1.5 MPa) drought stress treatment (Table 5.3).

5.3.6 Relations between WUE, carbon and nitrogen isotope composition and soluble sugars

In this experiment no significant correlation was observed between intrinsic WUE, instantaneous WUE with carbon isotope composition ($\delta^{13}C$) (Table 5.4). Also, no correlation was found between $\delta^{13}C$ and $\delta^{15}N$ (Table 5.4). There was a significant positive correlation (*P*

< 0.03) between leaf soluble sugars and intrinsic water use efficiency (Pn/g_s) (Figure 5.6) in the pistachio cultivars. There was no significant correlation between nitrogen isotope composition and intrinsic and instantaneous WUE under drought stress in the pistachio cultivars (Table 5.4).

Table 5.3: Mean values of intrinsic and instantaneous (inst.) water use efficiency (WUE, µmol CO_2 mol $^{-1}H_2O$), carbon isotope composition ($\delta^{13}C$, ‰) and nitrogen isotope composition ($\delta^{15}N$, ‰) in different osmotic drought stress levels (T, MPa) and three pistachio cultivar seedlings after two weeks osmotic stress. Values are means ± SE of nine replicates for both WUE and three for $\delta^{13}C$ and $\delta^{15}N$.

	T (MPa)	intrinsic WUE	inst. WUE	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Akbari	-0.1	46.96±4.79 b	5.57±0.27 a	-30.26±0.13 c	-3.27±0.47 a
	-0.75	89.57±12.83 a	8.28±1.08 a	-29.45±0.16 a	-2.73±1.12 a
	-1.5	87.76±12.82 a	8.39±1.02 a	-29.79±0.02 b	-3.68±1.13 a
Kaleghochi	-0.1	55.73±5.96 b	5.97±0.42 b	-30.11±0.20 a	-2.73±0.54 a
	-0.75	61.77±7.77 b	6.00±0.58 b	-29.47±0.10 a	-2.80±0.12 a
	-1.5	105.00±16.9 a	9.26±1.04 a	-30.49±0.59 a	-5.13±1.32 a
Ohadi	-0.1	51.83±8.07 a	6.00±0.55 a	-30.07±0.11 a	-2.96±0.93 a
	-0.75	86.31±9.15 a	6.98±0.66 a	-30.10±0.04 a	-3.94±1.24 a
	-1.5	90.58±14.12 a	8.29±0.99 a	-29.74±0.14 a	-4.80±0.97 a
ANOVA	C	ns	ns	ns	ns
	T	*	*	ns	ns
	C×T	ns	ns	ns	ns

Table 5.4: Overall correlation coefficients of intrinsic and instantaneous water use efficiency (WUE, µmol CO_2 mol $^{-1}H_2O$), carbon isotope composition ($\delta^{13}C$, ‰) and nitrogen isotope composition ($\delta^{15}N$, ‰), biomass (g), *Pn* (µmol $m^{-2}s^{-1}$) and soluble sugars in three pistachio cultivar seedlings in different osmotic drought stress levels (T, MPa) after two weeks osmotic stress.

Traits	intrinsic WUE	inst. WUE	$\delta^{13}C$ (‰)	$\delta^{13}N$ (‰)	Biomass (g)	*Pn*
inst. WUE	0.94 **					
$\delta^{13}C$ (‰)	-0.05^{ns}	-0.09^{ns}				
$\delta^{13}N$ (‰)	-0.29^{ns}	-0.26^{ns}	-0.13^{ns}			
Biomass (g)	-0.21^{ns}	-0.18^{ns}	-0.14^{ns}	-0.03^{ns}		
Pn	-0.61**	-0.48**	-0.12^{ns}	0.29^{ns}	0.42**	
Soluble sugar	0.28*	0.24*	0.03^{ns}	-0.06^{ns}	-0.27*	-0.50**

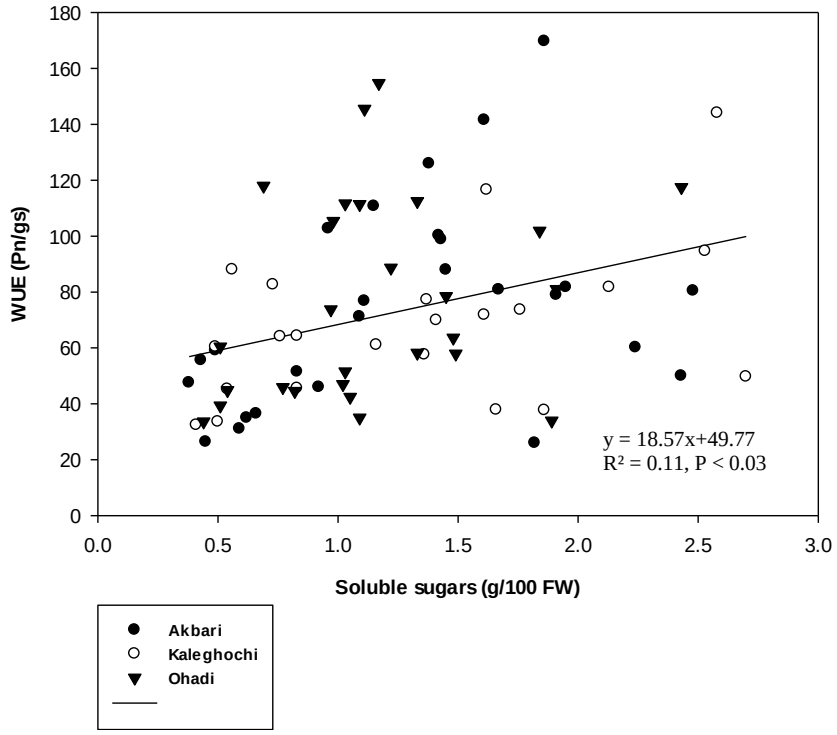


Figure 5.6: Relation between soluble sugars (g/100 fresh weight) and intrinsic water use efficiency (WUE, µ mol CO_2 mol $^{-1}H_2O$) in pistachio cultivars exposed to osmotic drought stress. Each symbol represents a value for every cultivar as shown in Table 5.4; the line represents the following linear relationship: y= 18.57x+49.77 ; R^2=0.11; n=27; *P* < 0.03.

5.3.7 Principal component analysis (PCA)

PCA analysis showed that six main components with eigenvalues > 1 together explained 95.3% of the total variation (Appendix 5- chapter 5).

PC1 explained 57.3% (Table 5.5) of the total data variation and had highly positive correlation with *Pn*, *gs*, YII, qP (all in recovery stage) and N and negative correlated with WUE (recovery stage). The second component (PC2) explained 13.9 % of the total variability and correlated positively with LWP (Recovery) and SDW. The third, fourth, fifth and sixth dimensions included total Chl (drought), carbon isotope composition, total Chl (recovery) and NPQ (drought) which accounted for 7.5%, 6.9%, 5.9% and 3.7% of the total variability, respectively.

Data obtained of PCA of control plants and plants submitted to increasing drought stress were visualized in a biplot analysis of PC1 and PC2, for the three cultivars (Figure 5.7). The angles between some measured parameters were acute (sharp angle). Therefore, these traits can be classified in two groups with high correlation. Group 1 indicated the traits related to photosynthesis rate and group 2 covered the traits related to osmotic adjustments.

The standard deviation of cultivars and osmotic drought stress treatments were shown in Figure 5.8. The results from this figure showed that the distances between normal (-0.1 MPa) and severe stress (-1.5 MPa) conditions for cultivars Akbari and Ohadi were biggest and smallest rates, respectively. Therefore, Akbari and Ohadi were less and more affected by drought stress and can be selected as susceptible and tolerant cultivars, respectively.

Table 5.5; Principle component analysis of measured parameters under different environmental conditions in three pistachio cultivars.

	Dimension					
Parameters	1	2	3	4	5	6
Hexose Dr.	-0.46	-0.667	-0.11	-0.299	0.278	0.276
Sucrose Dr.	-0.805	0.023	0.167	-0.404	-0.137	0.371
Starch Dr.	-0.796	0.435	0.139	-0.278	0.129	0.248
Hexose Re.	-0.816	-0.296	-0.127	-0.353	-0.313	-0.014
Sucrose Re.	**-0.918**	-0.075	0.148	0.075	-0.255	-0.043
Starch Re.	-0.889	0.381	0.132	0.175	0.072	-0.012
Proline Dr.	-0.726	-0.37	-0.025	-0.545	0.027	0.052
Proline Re.	-0.743	0.198	0.174	-0.374	-0.352	0.305
Total Chl. Dr.	0.283	0.33	**0.734**	0.12	0.209	0.375
Car. Dr.	-0.138	-0.575	**0.711**	0.103	-0.164	0.081
Total Chl. Re.	0.262	0.394	0.577	0.008	**0.639**	-0.042
Car. Re.	0.331	-0.851	-0.246	-0.042	0.148	-0.076
PFW	0.791	0.454	-0.193	-0.045	-0.267	0.082
SDW	0.2	**0.901**	-0.211	-0.089	-0.222	0.017
F_v/F_m Dr.	0.8	0.291	0.104	-0.324	0.263	-0.03
YII Dr.	0.863	0.211	-0.22	-0.188	-0.314	-0.008
Npq Dr.	0.125	-0.013	-0.549	0.178	**0.615**	**0.423**
qP Dr.	0.621	-0.261	-0.639	0.075	-0.082	0.293
F_v/F_m Re.	0.881	-0.086	-0.044	-0.374	0.099	-0.001
YII Re.	**0.956**	0.064	0.016	-0.034	-0.002	-0.026
Npq Re.	-0.772	0.166	-0.178	0.297	0.462	0.044
qP Re.	**0.923**	0.022	0.119	-0.023	0.087	-0.008
Pn Re.	**0.972**	-0.048	0.07	-0.121	-0.096	0.146
gs Re.	**0.96**	-0.112	0.147	-0.006	-0.06	0.083
Ci Re.	0.86	0.01	0.159	0.413	-0.186	0.008
WUE Re.	**-0.959**	-0.018	-0.117	-0.177	0.135	0.015
RWC Dr.	0.802	0.128	-0.032	0.29	-0.205	0.332
RWC Re.	**0.931**	-0.106	0.195	0.045	-0.209	-0.151
LWP Dr.	-0.65	0.67	-0.023	-0.205	-0.232	0.155
LWP Re.	-0.021	**0.915**	-0.357	0.014	0.116	-0.076
N	**0.972**	0.036	0.034	-0.227	-0.011	-0.022
Pn Dr.	0.875	-0.033	0.047	-0.281	0.074	0.215
gs Dr.	**0.959**	-0.028	0.093	-0.231	0.093	-0.037
Ci Dr.	**0.923**	-0.071	-0.008	-0.144	0.074	0.135
WUE Dr.	**-0.921**	0.109	-0.027	0.21	0.014	-0.22
C isotope	-0.291	-0.224	-0.012	**0.735**	-0.295	**0.465**
Hexose Dr.	-0.46	-0.667	-0.11	-0.299	0.278	0.276
Eigenvalue	20.613	5.021	2.728	2.499	2.137	1.325
Proportion (%)	57.257	13.947	7.577	6.942	5.935	3.681
Cumulative (%)	57.257	71.204	78.781	85.723	91.658	95.339

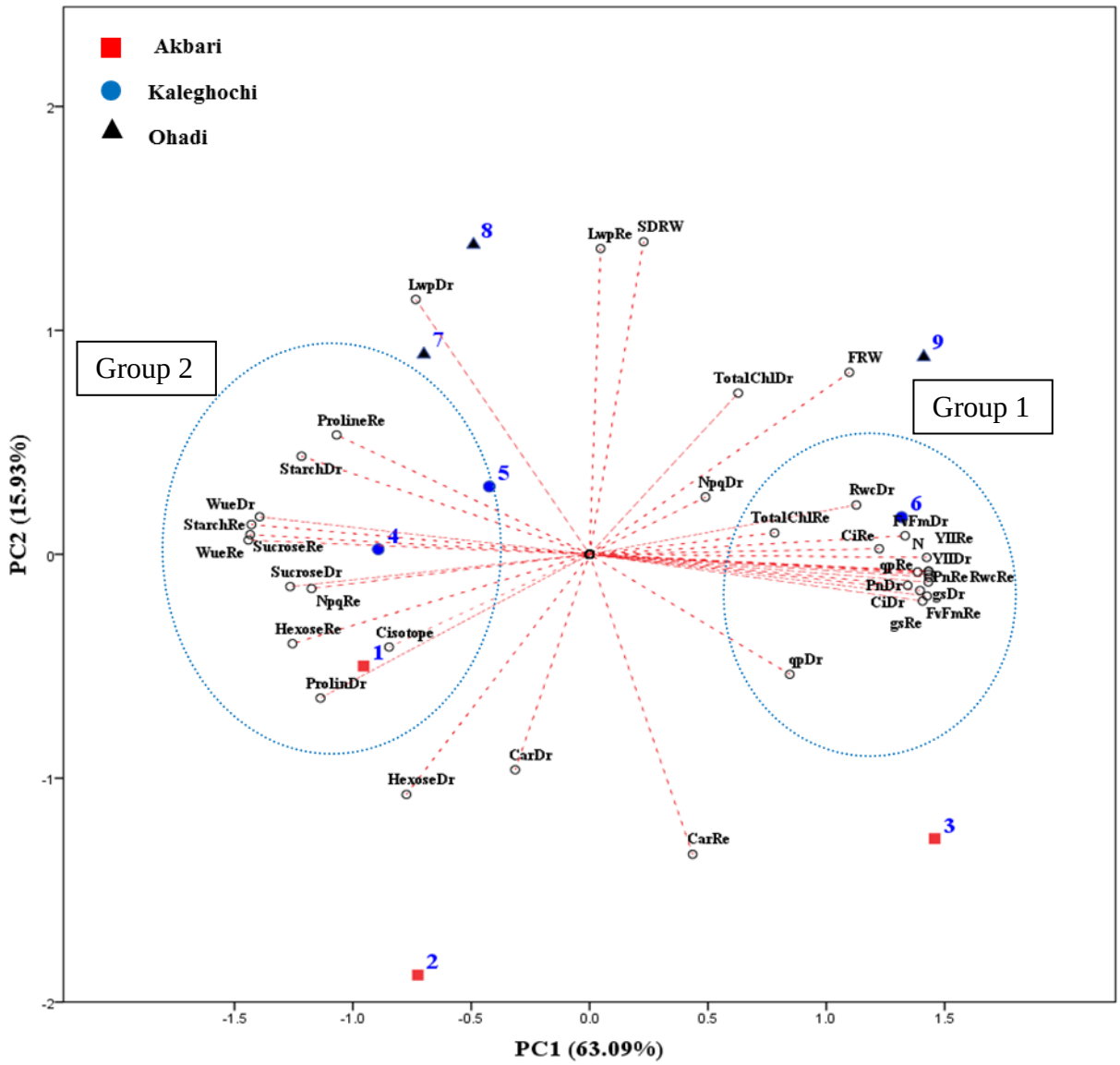


Figure 5.7: Biplot analysis of measured parameters and three pistachio cultivars under different environmental conditions. Numbers 1 to 3 represent the Akbari cultivar under three osmotic drought stress levels: -1.5 (MPa), -0.75 (MPa) and-0.1 (MPa), respectively; Numbers 4 to 6 represent the Kaleghochi cultivar; while Numbers 7 to 9 represent the Ohadi cultivar both under the same three osmotic drought stress levels.

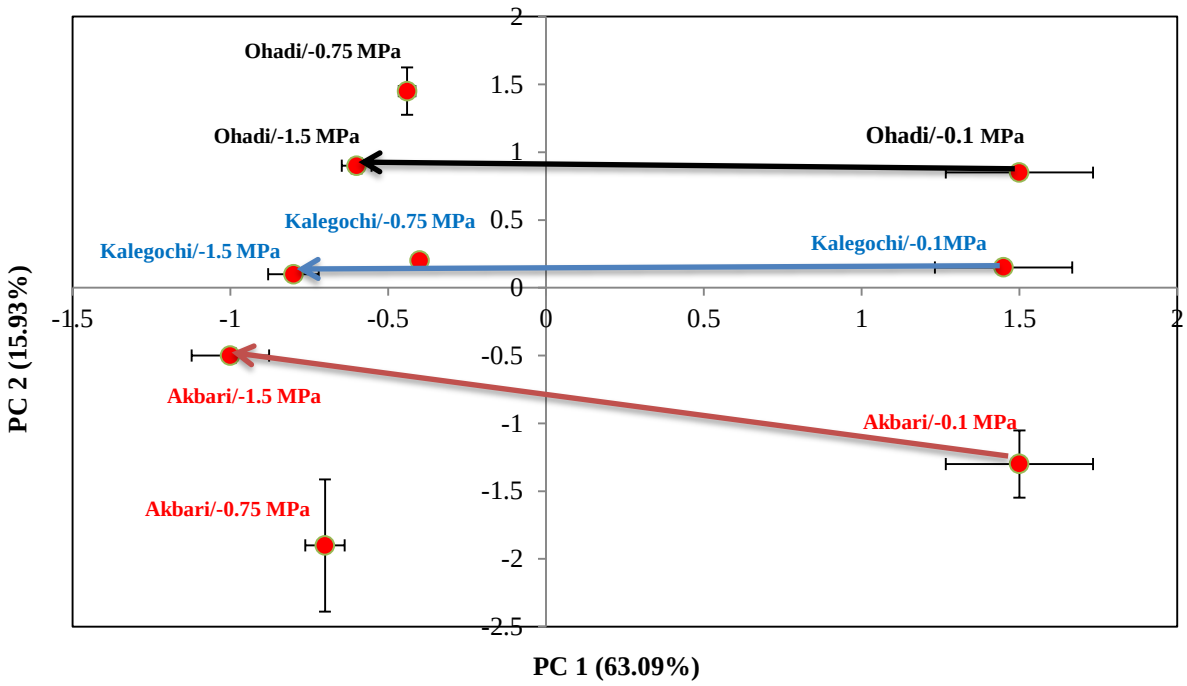


Figure 5.8: Standard deviations (SD) of three pistachio cultivars (Akbari, Kaleghochi and Ohadi) under three osmotic drought stress levels (-1.5 MPa, -0.75 MPa and -0.1 MPa) in Biplot analysis. Horizontal and vertical bars indicate SD for PC1 and PC2 (mean of nine replicates ± SD), respectively. Arrows in different colors show the distance between the control (-0.1 MPa) and the severe drought stress (-1.5 MPa) treatments in different cultivars (red- Akbari; blue- Kaleghochi and blank- Ohadi).

5.4 Discussion

In this study, we investigated the effect of osmotic drought stress induced by PEG on leaf water status as assessed by RWC, carbohydrates and stomatal characteristics and on WUE, nitrogen and carbon isotope composition of three important pistachio cultivars, i.e., *P. vera* Akbari, Kaleghochi and Ohadi.

RWC content is a good measure of plant water status, in terms of monitoring the physiological consequences of a cellular water deficit (Anjum et al., 2011; Blum, 2005; Farooq et al., 2009a). Our results showed that an osmotic drought stress of two weeks did not strongly affect RWC in leaves of the studied pistachio cultivars (Figure 5.4, A and B). These results are in contrast with previous reports on pistachio seedlings that indicated that RWC significantly decreased with drought stress treatments (Habibi and Hajiboland, 2013; Khoyerdi *et al.*, 2016; Panahi, 2009). However, our results are in agreements with

Behboudian et al. (1986), Hokmabadi et al. (2005) and Karimi et al. (2009) who reported that increasing salinity did not significantly affect RWC in different pistachio cultivars. Although the observed RWC indicated that plants could maintain relatively well their water balance by increasing the osmotic potential of the nutritive solution, decreasing Ψ_l of pistachio cultivars (Chapter 4).

In this study, leaf solute carbohydrates increased in response to both drought stress treatments (Table 5.1). This accumulation of low molecular-mass organic solutes, such as soluble sugars, proline or other amino acids, regulate the osmotic potential of cells to improve absorption of water under drought stress (Hessini *et al.*, 2009; Sánchez-Rodríguez et al., 2010; Zhang *et al.*, 2010). The three cultivars revealed differences in their accumulation of soluble sugars with increasing drought stress (Table 5.1) but the interaction effects were not significant. Accumulation of sucrose levels was 2; 1.6; and 2.2 times higher than controls for Akbari, Kaleghochi and Ohadi, respectively. This confirms the role of sucrose in osmotic adjustment under severe drought stress, which is consistent with previous observations in pistachio (Hajiboland et al., 2014) and other plant species (Arndt et al., 2001; Maraghni et al., 2014; Šircelj et al., 2005). Although slight increases for hexoses (glucose and fructose) were observed in all three cultivars, only for Kaleghochi fructose significantly increased under severe drought stress (-1.5 MPa, 1.8 x). Based on the results obtained in chapter 4 and this chapter, plant water status was maintained by leaf stomatal closure, which increased water use efficiency (WUE). Soluble carbohydrate accumulation helped to maintain leaf turgor and stomatal conductance for efficient intake of CO_2 and promotes the root's ability to take up more water. Consequently, all of these parameters affected leaf water potential of pistachio plants under drought conditions.

Surprisingly also leaf starch increased under drought stress (significantly for Ohadi; 2.7 times more than in control) contents (Table 5.1). This is in agreement with the results of Vilagrosa et al. (2003) who reported shoot starch concentration increased with increasing water stress in Aleppo pine (*Pinus halepensis* Mill.) seedlings. Moderate (-1.8 MPa) and strong (-2.2 MPa) water stress treatments exhibited the highest concentrations, accumulating 55% more starch than control plants. This response can be explained because growth is depressed earlier by drought (decrease in turgor pressure due to water loss) then are photosynthesis, or nutrient absorption (Trubat *et al.*, 2006; Vilagrosa et al., 2003). While, our results are in contrast with observations of starch decrease under drought stress in leaves of many other fruit trees (*Ziziphus mauritiana* and *Prunus persica*) (Arndt *et al.*, 2000). Thus,

for the above pistachio cultivars, osmotic adjustment is achieved by increasing the number of osmotically active molecules, i.e. proline (chapter 4) and soluble sugars, these increase was still present after a recovery of two weeks. This allows leaf turgor pressure to be maintained for a longer period during drought stress (Abbaspour et al., 2012a; Anjum et al., 2011; Arndt et al., 2000) and was also observed by Panahi (2009) on different pistachio species. Also Khoyerdi et al. (2016) found that pistachio cultivars maintained their turgor pressure through osmoregulation and increase of their sugar contents.

Controlling the plant water balance is also strongly regulated by its stomatal control, which is also influenced by density and morphology (Hetherington and Woodward, 2003). These characteristics are affected by the genotype and by environmental factors (Dong and Zhang, 2000; Munir et al., 2011). Changes in stomatal density are important as they can affect both the uptake of CO_2 and the rate of water loss (Alam, 1999). Stomatal density did not show clear changes in our experiments because all studied pistachio cultivars were exposed to drought stress for only a relatively short period (two weeks). Stomatal density can change clearly when plants are exposed to longer drought periods, i.e. leaf stomatal density can be adapted when new leaves are formed (Rouhi, 2007; Sanders and Arndt, 2012; Taiz and Zeiger, 2010). Severe drought stress significantly decreased stomatal length and width for Akbari plants. This was also observed in other edible pistachio cultivars under drought stress (Arzani et al., 2013), as well as in drought-stressed olive trees (*Olea europaea*) (Bosabalidis and Kofidis (2002). All drought stress treatments significantly decreased stomatal conductance, while severe drought stress declined the stomatal length and width in Akbari and the mild drought stress decreased stomatal density in Kaleghochi cultivar. There were no significant correlations between stomatal length, width and stomatal density with stomatal conductance (data not shown). A negative relationship, between stomatal length and width with stomatal density, suggests that stomata can adopt a coping mechanism to deal with excessive drought stress (data not shown). This is also observed in forest tree species where species with larger stomata were slower to close and more sensitive to increasing drought (Hetherington and Woodward, 2003). However, also genotypic differences were observed as stomatal density for the studied cultivars was lower than those reported for wild pistachio rootstocks (Ranjbarfordoei et al., 2002) and other edible cultivars (Arzani et al., 2013).

Under drought stress plants change to a more efficient water use by partial closure of their stomata. Indeed, both instantaneous WUE (*Pn*/E) and intrinsic WUE (*Pn*/*gs*) increased with increasing osmotic stress (Table 5.3), as *Pn*, E and *gs* decreased by increasing drought stress

(chapter 4). Enhanced WUE was still present after a 2-week recovery phase (Table 4.2). Our results are consistent with the report of Behboudian et al. (1986) on pistachio (*P. vera* L.), and Arndt et al. (2001) on common jujube (*Ziziphus rotundifolia),* Rouhi et al. (2007) on almond (*Prunus* spp.) rootstocks. However, in two wild pistachio species (*P. mutica* and *P. khinjuk*) photosynthesis and transpiration evolved in a similar way under increasing osmotic stress (up to -1.4 MPa) resulting in an unaffected WUE (Ranjbarfordoei et al., 2000). This difference may be related to genetic effects of the different pistachio species. Another aspect of genetic differences is found in the absolute values of the instantaneous WUE: we found on average 7 to 7.3 µmol CO_2 mol^{-1} H_2O while 18.6 to 23.4 µmol CO_2 mol^{-1} H_2O was found for *P. mutica* and *P. khinjuk* (Ranjbarfordoei et al., 2000) indicating a better drought tolerance of the wild species. We also observed a strong negative correlation between the intrinsic WUE and photosynthesis (Table 5.4). On a long term this would also result in a negative correlation between WUE and productivity (Li, 1999). Yet, this correlation was not observed in our 2-weeks experiment.

This is the first report to our knowledge that investigates effects of drought stress treatments on carbon and nitrogen isotope composition in pistachio seedlings. The observed leaf $\delta^{13}C$ of control plants are within the range of -30.07 to -30.26‰, these values are also reported for different *Eucalyptus* populations (Li, 1999). The applied moderate (-0.75 MPa) and severe (-1.5 MPa) drought stress treatments did not result in any change of the leaf $\delta^{13}C$ despite the observed changes in WUE, this irrespective of the cultivar. Hokmabadi et al. (2005), however, found carbon isotope discrimination (Δ) decrease with increasing salinity in leaves, stems and roots pistachio seedlings though RWC was not affected. Differences might be attributed to the time scale of observations, two weeks in our experiment and four to eight weeks in the salt stress experiment. However, Pascual et al. (2013) showed leaf carbon isotope ratio was not significantly affected by either irrigation or nitrogen treatments with different rootstocks of adult peach trees (*Prunus persica*).

In general, no significant correlations between $\delta^{13}C$ with $\delta^{15}N$ and *Pn* (Table 5.4) and no correlation between intrinsic and instantaneous WUE with carbon isotope composition (Table 5.4) were found. This is in agreement with results on sunflower (*Helianthus* spp.) (Lambrides *et al.*, 2004) and agrees with the model of Farquhar and Richards (1984) developed for wheat, though our results are in contrast with Arndt et al. (2001) in *Ziziphus rotundifoli* and de Souza et al. (2005) in grapevines. The lack of a significant correlation between intrinsic WUE and carbon isotope composition for the studied pistachio cultivars

exposed to drought stress (Table 5.4), indicates that this relation may not be used to determine pistachio cultivars with appropriate WUE through leaf carbon isotope composition in short term experiments.

Yousfi et al. (2012a) reported different plant parts may have a different reaction for $\delta^{15}N$, when plants are exposed to drought stress conditions. Root $\delta^{15}N$ increased under drought stress condition; in contrast drought treatments significantly decreased shoot $\delta^{15}N$ in durum wheat (*Triticum durum*). In our experiment, leaf $\delta^{15}N$ values under drought stress treatments were, however, none significant compared to controls. A more negative shoot $\delta^{15}N$ therefore suggests reduced nitrate assimilation under increasing osmotic stress which might be reflected in reduced leaf nitrogen content as found in chapter 4. Isotope fractionation of nitrogen may occur during uptake from the medium into root cells, or during subsequent enzymatic assimilation into other N forms. Further fractionation may also occur if biochemical components of varying isotopic composition are lost through translocation, efflux, or emission (Evans, 2001; Pritchard and Guy, 2005). Furthermore nitrogen deficiency in drought stress conditions will result in the reduction of photosynthetic rate, which would affect growth rate and biomass production.

According to data of Table 5.5, the PC1 can be considered as a component related to photosynthesis and WUE. In other words, this component was able to separate the plants with high photosynthesis and WUE under different drought stress conditions. The second component can be named as LWP and SDW dimension and it separates the drought tolerant cultivars with high growth and water retention capacity.

The results indicated that biplot of PCA clearly separated three cultivars which were grown under three water stress conditions. Thus, selection of cultivars that have high PC1 and PC2 is suitable for different stress conditions because these cultivars have a high photosynthesis rate along with high growth and water retention capacity. From the biplot, cultivars Ohadi (9) with high PC1 and PC2 had high photosynthesis rate along with high growth and water retention capacity under non stress conditions. Biplot depicted that cultivar Ohadi (8) as compared with Akbari (2) and Kaleghochi (5) represented higher PC1 and PC2 under moderate drought stress condition (-0.75 MPa) and were selected as tolerant cultivar in this conditions.

Under severe stress condition (-1.5 MPa), Ohadi (7) with higher PC1 and PC2 was selected as tolerant cultivar compared to Akbari (1) and Kaleghochi (4). Generally, the biplot indicated Ohadi as more tolerant and Akbari as more susceptible cultivars, respectively, under drought stress conditions.

5.5. Conclusion

Under osmotic stress conditions, pistachio cultivars developed an active drought tolerance mechanism to cope with water deficiency. The present study has shown that drought stress treatments increased soluble sugars and starch levels in our pistachio cultivars, although relative water content was only moderately affected. WUE increased with the higher content of osmotically active molecules, i.e. soluble sugars and proline, this increase was still present after a recovery of two weeks. The observed lower stomatal density (Kaleghochi) and/or reduced stomatal sizes (Akbari) can be considered as an anatomical adaptation at leaf level to reduce its transpiration.

The applied moderate and severe drought stress conditions did not result in any change of the leaf $\delta^{13}C$ despite the observed changes in WUE. This indicates that this relation may not be used to determine pistachio cultivars with appropriate WUE through leaf carbon isotope composition. In contrast leaf $\delta^{15}N$ indicated reduced nitrogen assimilation under increasing drought stress.

According to the results of this experiment (Chapter 4 and 5), Akbari seems to be a drought susceptible cultivar, while Kaleghochi and Ohadi seems to be drought tolerant cultivars.

Chapter 6. **General conclusions and perspectives**

6.1 General conclusion

Water resources in arid and semi-arid regions are limited and are subjected to increasing competition between agricultural, domestic and industrial uses. Climate warming will even increase the need for irrigation. However, increased evapotranspiration rates and reduced rainfall will limit water resources for irrigation. The progression of drought is increasingly threatening agricultural production.

Arid and semi-arid climates prevail in approximately 80% of Iran's land surface. Water deficiency and poor water quality are the most common factors that affect the quality and quantity of the crop production in these areas. Iran is the largest pistachio producer and exporter in the world. However, high salinity of agricultural water and inadequate irrigation are the main restrictions which farmers are facing (Sedaghat, 2010). Recently, pistachio orchards productivity has declined and also Iran's share in the global pistachio market has decreased significantly (Abdolahi, 2016; Sedaghat, 2010). Because arid and semi-arid areas are not suitable to produce other crops in profitable way, pistachio cultivation remains the only crop for farmers.

Increasing pistachio yield (quality and quantity) is one of the most important strategies in order to maintain the country's share in global markets. There are two ways to achieve a higher pistachio production: i) extension of pistachio plantations in new areas; and ii) increasing pistachio nut production per hectare. The first way is the most common in Iran. Local farmers use the traditional rootstock (*P. vera* cv. Badami) to establish new pistachio orchards in new areas although no advanced rootstock strategies are used in Iran. Another strategy includes searching for more tolerant genotypes against abiotic and biotic stresses like drought, salinity, cold, heat, pests and diseases. In this PhD research we have focused on drought acclimation strategies in pistachio cultivars. Human and natural selection lead to a wide genetic diversity among cultivated and wild species of pistachios. The search for rootstocks and cultivars which are more tolerant to abiotic factors is urgent and crucial as breeding for drought tolerance is a long-term process in fruit crops.

According to the results obtained in the rootstock experiment, there were some considerable variations among pistachio rootstocks evaluated (Table 6.1 and appendix 6-chapter 6). Taking all ecophysiological and biochemical parameters obtained in this study into consideration, it can be concluded that Terebinthus (*P. terebinthus*) rootstock is more tolerant to lower water availability in comparison with the other two evaluated rootstocks namely Badami and Sarakhs (*P. vera*) that can be considered to be moderately drought

tolerant and sensitive to drought, respectively. Consequently, pistachios growers have to consider the use of this rootstock instead the others that may be more susceptible to drought condition in new plantation areas.

Table 6.1: Overview of the changes of all measured parameters under severe drought stress treatment (-1.5 MPa) compared to corresponding control (-0.1 MPa) plants of the drought intermediate, tolerant and susceptible pistachio rootstocks under drought stress conditions. Parameters that do not have unit, indicated by minus sign (-). Significant decrease, significant increase and non- significant changes are shown with down arrow (↘), up arrow (↗) and n.s., respectively.

			Pistachio rootstocks		
			Badami	**Terebinthus**	**Sarakhs**
Main parameters	**Sub-parameters**	**Unit**	**Drought intermediate**	**Drought tolerant**	**Drought susceptible**
Chlorophyll fluorescence parameters	F_v/F_m	-	n.s.	n.s.	n.s.
	YII	-	↘	↘	↘
	NPQ	-	n.s.	n.s.	n.s.
	qP	-	↘	↘	↘
Growth parameters	PFW	g	n.s.	↘	n.s.
	PDW	g	n.s.	↘	n.s.
	LDW	g	↘	↘	n.s.
	RDW	g	n.s.	n.s.	n.s.
	R/S ratio	ratio	↗	n.s.	n.s.
	LA	Cm^2	↘	↘	n.s.
	SEL	Cm	↘	↘	↘
	shedding	g	n.s.	n.s.	n.s.
Nutrient elements	P	%	↘	n.s.	↘
	K	%	n.s.	n.s.	n.s.
	Ca	%	↘	↗	n.s.
	Mg	%	↘	n.s.	n.s.
	Fe	ppm	n.s.	n.s.	n.s.
	Zn	ppm	n.s.	↗	n.s.
Isotope composition	$\delta^{13}C$	‰	n.s.	n.s.	n.s.

F_v/F_m - maximum quantum yield of PSII
YII- effective quantum yield of PSII electron transport
NPQ- non-photochemical quenching of chlorophyll fluorescence
QP- photochemical quenching of chlorophyll fluorescence
PFW- plant fresh weight
PDW - plant dry weight (biomass)
LDW- leaf dry weight
R/S- root /shoot ratio
LA- leaf area
SEL- stems elongation
P- phosphorous
K- potassium
Ca- calcium

Mg- magnesium
Fe- iron
Zn - zinc
$\delta^{13}C$- carbon isotope composition

Drought stress had a suppressing effect on the studied parameters in all pistachio cultivars including Akbari, Kaleghochi and Ohadi, although its degree depended on the cultivar and stress intensity. Some remarkable variation was observed between the three studied cultivars (Table 6.2 and appendix 7- chapter 6). The observed responses lead to a better understanding of drought-tolerant mechanisms in pistachio cultivars.

Based on our results obtained in chapters 4, 5 and mentioned in Table 6.2, Ohadi pistachio, the most common cultivar in most pistachio plantation areas in Iran (Kerman province), shows better capability to cope with drought conditions than Kaleghochi and Akbari which have only recently been distributed in these areas. This recommendation is also supported by drought tolerance of Ohadi cultivar under field conditions that are not irrigated for long times (more than 5 years). These results can be important when farmers want to extend plantation areas for new pistachio orchards in arid and semi-arid regions. Therefore, farmers are recommended to use of this cultivar instead the others that may be more susceptible to drought condition in new plantation areas.

Table 6.2: Overview of the changes of all measured parameters under severe drought stress treatment (-1.5 MPa) compared to corresponding control (-0.1 MPa) plants of the drought susceptible, intermediate and tolerant pistachio cultivars under drought stress conditions... Parameters that do not have unit, indicated by minus sign (-). Significant decrease, significant increase and non- significant changes are shown with down arrow (↘), up arrow (↗) and n.s., respectively.

			Pistachio cultivars		
			Akbari	**Kaleghochi**	**Ohadi**
Main parameters	**Sub-Parameters**	**Unit**	**drought susceptible**	**drought intermediate**	**drought tolerant**
Gas exchanges parameters	*Pn*	µmol $m^{-2}s^{-1}$	↘	↘	↘
	gs	mol H_2O $m^{-2}s^{-1}$	↘	↘	↘
	Ci	µmol CO_2 mol^{-1}	↘	↘	↘
	E	mmol (H_2O) $m^{-2}s^{-1}$	↘	↘	↘
	WUE	Pn/g_s	↗	↗	↗
Chlorophyll fluorescence parameters	F_v/F_m	-	↘	n.s.	n.s.
	YII	-	n.s.	n.s.	n.s.
	NPQ	-	↘	n.s.	n.s.
	qP	-	↘	n.s.	n.s.
	ETR		n.s.	n.s.	↘
Photosynthesis pigments	Chl *a*	µg/g FW	↘	↘	n.s.
	Chl *b*	µg/g FW	↘	↘	↘
	Car	µg/g FW	n.s.	n.s.	n.s.
Growth parameters	PFW	g	↘	n.s.	n.s.
	PDW	g	n.s.	n.s.	↘
	LDW	g	↘	n.s.	↘
	RDW	g	n.s.	n.s.	n.s.

	R/S ratio	ratio	n.s.	n.s.	n.s.
	SEL	Cm	↘	↘	↘
Nutrient elements	N	% DW	↘	↘	↘
Water status parameters	LWP	MPa	↘	↘	↘
	RWC	%	n.s.	↘	n.s.
Osmolyte accumulation	Proline	µmol g^{-1} FW	↗	n.s.	n.s.
	Glucose	g/100 g FW	n.s.	n.s.	n.s.
	Fructose	g/100 g FW	n.s.	↗	n.s.
	Sucrose	g/100 g FW	↗	↗	↗
	Starch	g/100 g FW	n.s.	n.s.	↗
Stomatal characteristics	SL	µm	↘	n.s.	n.s.
	SW	µm	↘	n.s.	n.s.
	SD	-	n.s.	n.s.	n.s.
Isotope compositions	$\delta^{13}C$	‰	↗	n.s.	n.s.
	$\delta^{15}N$	‰	n.s.	n.s.	n.s.

Pn- net photosynthesis
g_s- stomatal conductance
C_i- intercellular CO_2
E- transpiration rate
WUE- water use efficiency
F_v/F_m - maximum quantum yield of PSII
YII- effective quantum yield of PSII electron transport
NPQ- non-photochemical quenching of chlorophyll fluorescence
QP- photochemical quenching of chlorophyll fluorescence
ETR- electron transport rate
Chl a- chlorophyll a
Chl b- chlorophyll b
Car- carotenoid
PFW- plant fresh weight
PDW - plant dry weight (biomass)
LDW- leaf dry weight
RDW- root dry weight
R/S- root /shoot ratio
SEL- stems elongation
N- nitrogen
LWP- leaf water potential
RWC- relative water content
SL- stomata length
SW- stomata width
SN- stomatal number
$\delta^{13}C$- carbon isotope composition
$\delta^{15}N$- nitrogen isotope composition

Our results demonstrate that different pistachio genotype use different mechanisms to deal with drought stress. Evaluated pistachio rootstocks (Terebinthus (*P. terebinthus*) and Badami and Sarakhs (*P. vera*)) and pistachio cultivars (Akbari, Kaleghochi and Ohadi (*P. vera*)) seedlings apply drought avoidance (the maintenance of turgor through increased root system efficiency and water loss reduction through reduced stomatal conductance) (Levitt, 1980; Mitra, 2001; Turner, 1986a), and drought-tolerance (maintenance of turgor through osmotic adjustment and metabolite accumulation) (Chaves and Oliveira, 2004; Van Damme, 1990) mechanisms. These mechanisms (drought avoidance and tolerance) were also shown for other pistachio cultivars such as; Badami-riz, Ghazvini, Sarakhsi, Ahmadaghaii and Abareghi (Bagheri et al., 2012; Habibi and Hajiboland, 2013; Khoyerdi et al., 2016) and *Pistacia mutica* and *Pistacia khinjuk* rootstocks (Ranjbarfordoei et al., 2002). Although, drought avoidance and drought tolerance have also been reported for other arid and semi-arid crops that were exposed to drought stress conditions such as; *Ziziphus lotus* (Maraghni et al., 2014), *Olea europaea* L. (Boussadia *et al.*, 2013) and *Prunus scoparia* (Rouhi et al., 2007).

6.2 Perspectives for future research

In Iran, there will be continuous research efforts by government research centers to introduce more tolerant pistachio cultivars and rootstocks to abiotic stress conditions. Because of the high pistachio genetic diversity in Iran (more than 150 cultivars and 3 species), and limited information about foreign rootstocks, only a few farmers are interested to use foreign rootstocks (those which are not originally from Iran) in their new orchards. Increased knowledge about the drought tolerance of both domestic and foreign pistachio rootstocks is critical for farmers to select the most appropriate rootstock.

This experiment was done under glasshouse conditions with exposing the plants only under the drought stress condition, but influences of others abiotic and biotic stress conditions such as; salinity, hot stress (over temperature), cold, nutrition elements, heavy metal, pests and diseases were not determined. According to the results of this study most of physiological responses to drought stress conditions are accompanied with the changes in biochemical characteristics, while there was no combined instrument to measure these parameters at the same time.

The period of drought stress (two weeks) was insufficient to provide real response for some measured parameters especially for gas exchange and chlorophyll florescence characteristics, osmolyte accumulations and carbon and nitrogen isotope compositions. On

the other hand, the recovery stage (two weeks) also was inadequate for drought exposed seedlings to be restored to a state similar to untreated conditions. Different pistachio seedlings were evaluated under juvenile stage, so recommendation about the effects of drought stress treatments on flowering time, fruit set, fruit ripening, yield qualitative and quantitative characteristics and alternate bearing habit for adult trees are not expected.

Due to the high experimental efforts, time limitations and no previous planning in this Ph.D. study, we did not evaluate the drought tolerance and drought susceptibility of pistachio rootstocks and cultivars by molecular markers that give better and more strategies. *P. terebinthus* rootstock is not originated from Iran region that is why there is limitation of scientific resources to indicate the response of this species to most biotic and abiotic stresses that are very common in Iran's pistachio production regions.

The most perspectives for future research are described as follows;

- ❖ According to the evaluated parameters in this study including morphological, ecophysiological and biochemical parameters, we were able to select the most sensitive parameters to evaluate and screen interesting cultivars and rootstocks. Terebinthus (*P. terebinthus*) species and the native pistachio Ohadi (*P. Vera* L.) cultivar, now well-studied during the first (Chapter 3) and second (Chapter 4 and 5) drought stress experiments, respectively, could be used as the recommended rootstocks for new pistachio orchards.
- ❖ Our first experiment revealed that effective screening trials to study drought tolerance of pistachio rootstocks i, e. Badami (*P. vera* L.) Terebinthus (*P. terebinthus*) and Sarakhs (*P. vera* L.) (Chapter 3) can be based on biomass parameters (SDW, PFW, PDW, LDW, R/S ratio), F_v/F_m and YII as suitable indicators for the screening and classification of pistachio rootstocks under different drought stress conditions. The results obtained in our second experiment revealed that efficient screening procedures for evaluating pistachio cultivars i.e. Akbari (*P. vera* L.), Kaleghochi (*P. vera* L.) and Ohadi (*P. vera* L.) (Chapter 4 and 5) could be based on the *Pn*, *gs*, YII, N, LWP and SDW as suitable indicators to classify pistachio cultivars under different drought stress conditions. $\delta^{13}C$ and N values have, to our knowledge, not been used earlier as drought stress parameters in pistachio, although they responses are frequently used in salt stress response research in pistachio (Afrousheh et al., 2010; Hokmabadi et al., 2005).
- ❖ Our study clearly illustrates that variations in the drought tolerance of pistachio cultivars is associated with variation in different physiological traits such as chlorophyll fluorescence and metabolite compositions. Consequently, the latter physiological

parameters also need to be investigated to understand how drought tolerance is established in pistachio cultivars and to discriminate their relative drought-tolerant levels. For a good understanding, combined physiological and biochemical traits are required to screen drought tolerance of pistachio genotypes rather than analyzing a single trait. The developed toolbox of chlorophyll fluorescence and metabolites, combined with photosynthesis and respiration would further provide useful information on abiotic tolerance in pistachio.

- Although our study gives a good insight into the responses of pistachio rootstocks and cultivars to drought stress, experiments were conducted in controlled glasshouse conditions where the interaction with other abiotic variables was limited. Future research should therefore study the behavior of the same rootstocks and cultivars in field conditions and interaction effects of rootstocks and cultivars with high light intensities and/or elevated temperature on biochemical and physiological parameters.
- It will also be interesting to investigate the plant's adaptive strategy if they are exposed to the same or even more severe water deficit conditions during a longer period (e.g. 4-8 weeks) followed by a longer recovery period (4-8 weeks) in both tolerant and sensitive pistachio genotypes.
- Although during the juvenile stage, a clear distinction between tolerance levels can be made, further research should also investigate the effect of drought stress on the production potentiality of the same adult pistachio cultivars. Such research must involve assessing the impact of drought stress on flowering, fruit development stages, ripening and more specifically, on kernel growth of developing young fruits during summer which could adversely affect yield under water scarcity.
- More in-depth studies could assess the impact of drought on pollen tube growth, stigma receptivity and fruit set, as fruit set percentage decrease following drought stress during the flowering period. A better understanding of precocious flowering under moderate drought stress is relevant in relation to spring frost damage which affects productivity in adult trees.
- The study of yield components (amount of nuts per tree/ha, kernel percentage) is economically important as it will allow identifying the reduction in production of nuts caused by drought in tolerant and susceptible cultivars. In addition, not only total production but also fruit quality parameters such as percentage of split nuts, blank nuts,

fruit color and average fruit weight (g) should be evaluated under drought stress conditions.

- It was shown that the three investigated rootstocks (chapter 3) clearly differed in their response to drought. As a result, due to the drought avoidance mechanism of Badami by leaf shedding (Chapter 3), this rootstock seems to be less suited as a rootstock compared to Terebinthus, because it might cause leaf shedding in scions (Chatterjee, 1979; Taiz and Zeiger, 2010). Although, and as an alternative for the commonly used Terebinthus, Badami also seems to have some potential as rootstock. However, further research is needed to support this statement.
- We suggest examining the effects of exogenous application of osmolytes (such as proline) on pistachio plant species/cultivars growing under stress conditions to enhance their tolerance on adult tress.
- Identifying differences among the considered genotypes in regard to nut production under drought stress conditions would be an important step forward in the development of cultivars with a reduced alternate bearing intensity.
- Field studies on the interaction between soil nutrients (Ca, K and Zn) and drought stress interactions are recommended.
- Results recommend further research on long-term drought experiments and water requirements of pistachio plants in the field. Moreover, further research on drought stress, scion and rootstock compatibility are recommended.
- A pistachio breeding program is needed to develop new cultivars/rootstocks for Iran's pistachio industry. The program can begin by developing tolerant rootstocks and cultivars to abiotic stress such as drought (Hasheminasab and Assad, 2017), salinity, cold and excessive temperature during the juvenile stage. This breeding program should be followed by evaluation of biotic stress against very common pistachio diseases (*Phytophthora* spp., *Aspergillus* spp. and V*erticilium* spp.) and pests (*Agonoscena pistaciae*, *Kermania pistaciella*, and *Brachenema* spp.). Later, pistachio rootstocks and cultivars selected during the juvenile stage should be tested in field conditions against different biotic and abiotic stress. Finally, the best cultivars and rootstocks combinations should be promoted as a commercial combination with high drought tolerance and good product performance. Combinations are best tested in the same field conditions. There are possibilities to use three domestic rootstocks (*P.vera*, *P. mutica* and *P. khinjuk*) and one foreign rootstock (*P. terebinthus*) combined with ten commercial cultivars (Akbari, Kaleghochi, Ahmadaghai, Ohadi, Momtaz, Fandoghi 48, Abbasali, Shahpasand, Kalebozi

and Badami-Safid) as suitable combinations for new pistachio plantation areas in Iran. We will need to evaluate the drought stress responses under field conditions for all rootstock x scion combinations.

- Terebinthus pistachio rootstock used in this experiment, is not originally from Iran and research on Iran's biotic (disease and pests) and abiotic stress (salinity, cold and over temperature) responses for the latter rootstock, is scarce. Therefore, researchers have to evaluate and compare this rootstock with Iranian common pistachio rootstocks such as *P.vera* cvs Badami, Sarakhs and Ghazvini, *P. mutica* and *P. khinjuk* that is needed to examine against others biotic and abiotic parameters except drought stress.
- For any breeding program, the choice of parents to be used in the crossing program is of paramount importance and constitutes the basis for the success. Our results indicate that Terebinthus and Ohadi can be used directly as suitable rootstock and cultivar/scion, respectively, to extend pistachio plantation in arid and semi-arid regions of Iran and other countries. These genotypes also can be used as ideal parents for improvements of pistachio drought tolerance (major objective in pistachio breeding programs in Iran) in the crossing program. Although, the photosynthesis parameters can be used as suitable indices to screen drought tolerant pistachio cultivars in pistachio breeding programs, application of molecular markers is expected to facilitate breeding programs. A more fundamental perspective could be provided by studying molecular markers for effective genes that induce drought tolerance in pistachio cultivars. Effective genes could be revealed by e.g. genetic linkage mapping and qualitative trait loci (QTL).

6.3 Recommendations for farmers, researchers, and government

Farmers are advised to use the mentioned recommended rootstock and cultivar (*P. terebinthou* and *P. vera* L. cv. Ohadi.) to extend pistachio plantation areas in new orchards. Even in harsh drought stress conditions, it is best to use the combination of Terebinthus as a rootstock and Ohadi as scion grafted on the latter rootstock in these areas.

There are laws on plant material exchange between countries, limiting access to pistachio genetic material to researchers. Governments should therefore strive to establish an international agreement on plant material exchange. In arid regions, one of the priorities should be to encourage farmers to invest in pistachio planting.

Over the past few years, climate change in Iran has caused substantial weather fluctuations, affecting many plant species. Some plant species such as pistachio, can more

easily adapt to climate change because of the wide range of their optimum growth conditions. This adaptation has led to the extension of plantation areas, where in some cases of other species these climatic changes have resulted in the reduction of plantation areas. According to what has been observed in pistachio plantation areas in Kerman province (Iran), climatic change is forcing large-scale replacement of sensitive cultivars by cultivars that are more resistant against or adapted to drought and salinity conditions. Because tolerant pistachio cultivars could adopt themselves with changes of climate more easily. The Iranian agricultural extension service plays a very significant role in enhancing agricultural production in the country. At present, extension should focus on the replacement of old pistachio cultivars and rootstocks with new genotypes and tolerant cultivars. Increased extension staff numbers and budgets are needed to identify and distribute new pistachio tolerant rootstocks and cultivars to the different pistachio growing region of Iran.

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Curriculum Vitae

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1987-1991	**Bachelor in Horticulture Science:** Faculty of Agriculture, Guilan University. Rasht, Iran.

Papers Presented in International Conference

- **Esmailpour, Ali**. 1998. Distribution, use and conservation of pistachio in IRAN. Submitted to: Pistachio genetic resources. Irbid, JORDAN.
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- **Esmailpour, A**. and A. R. Saberi. 2004. Identifying pistachio genetic resources in Sistan and Baluchistan province. First native knowledge and hereditary natural sources and agriculture resources gathering. 12-13 Oct. Zahedan, IRAN.
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Member of National & International Societies

- Member of international society of horticulture science (ISHS)
- Member of Iranian society of horticulture science

Research M.Sc. & Ph.D. Students Supervised

- Saba, M.1998. Designing and manufacturing method of manure dispersal machine. M.Sc. thesis. Tehran University, Tehran, IRAN.

Courses Taught

- Pistachio production 1 (undergraduate)
- Pistachio production 2 (undergraduate)
- Horticulture (undergraduate)

- Small Fruits in horticulture (undergraduate)

Books and booklets in Persian

- Panahi, B.; Esmailpour, A.; Farbood, F.; Moazenpour, M. and H. Farivar-Mehin. Pistachio handbook (Planting, processing and harvesting) 2002. Agriculture training publication. ISBN 964-6598-70-6.
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- **Esmailpour, A.** 2011. Pruning of pistachio trees. In. Hokmabadi, H. (eds). Diagnosis of environmental and none-environmental damaging factors incoming to pistachio product. Agriculture education and extension publication. ISBN 978-600-5898-72-2. 314 pages.
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Research Interest

- Rootstock and scion
- Training and pruning
- Cultivars
- Pollination and fruit set
- Physiology

Research Paper Publications in National & International

Journals

- **Esmailpour, A** and M. Rahemi. 2001. Effects of heading back on branching, flowering and pistachio yielding. Iranian journal of horticultural science and technology**.**Vol.1, Nos.1&2.
- Shibani A.; A. V. Azghandi; **A. Esmailpour** and N. Rohani; 1997; Characteristics of selected pistachio cultivars in Iran. Acta horticulture (1997) No. 144, 125-128.
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- **Esmaeilpour, Ali;** Van Labeke, Marie-Christine; Samson, Boeckx; Van Damme, Patrick. *2015.* Osmotic stress affects physiological responses and growth characteristics of three pistachio cultivars. *Acta Physiologiae Plantarum* 37(6):1-14.
- **Esmaeilpour, Ali;** Van Labeke, Marie-Christine; Samson, Boeckx; Ghafaripour, S.; Van Damme, Patrick. *2015.* Comparison of biomass production-based drought tolerance indices of pistachio (*Pistacia vera* L.) seedlings in drought

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- **Esmaeilpour, Ali;** Van Labeke, Marie-Christine; Samson, Boeckx; Boeckx, Pasccal; Van Damme, Patrick. 2016. Variation in biochemical characteristics, water status, stomata features, leaf carbon isotope composition and its relationship to water use efficiency in pistachio (*Pistacia vera* L.) cultivars under drought stress condition. Scientia Horticulturae 211, 158-166.

Scientific awards and distinctions

1 - Superior researcher at Iranian Pistachio Research Institute in 2002 – Research activities. Rafsanjan, IRAN

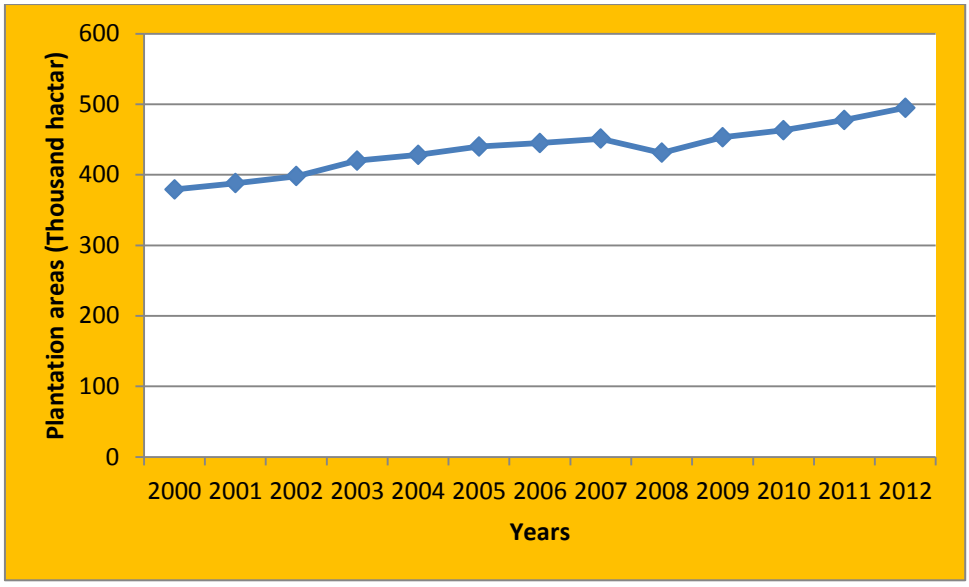
2 - Superior researcher at Iranian Pistachio Research Institute in 2003 – Research activities. Rafsanjan, IRAN

3 - Superior researcher at Iranian Pistachio Research Institute in 2004 – Research activities. Rafsanjan, IRAN

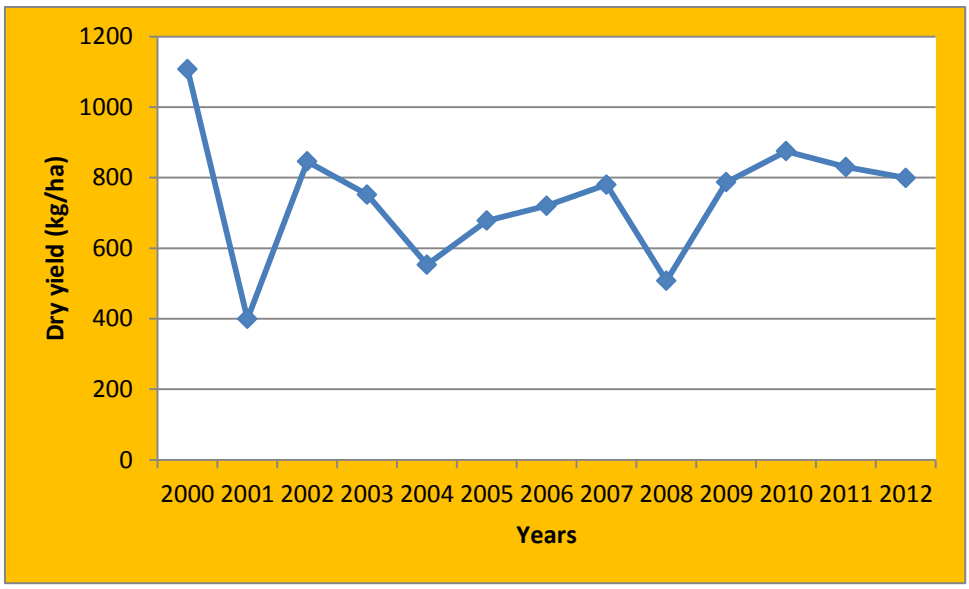
4 - Superior researcher at Research, education and extension organization in 2003 – Research activities. Tehran, IRAN

5 - Superior researcher at Agriculture minister in 2004 – Research activities. Tehran, IRAN

Appendices



Appendix 1–chapter 1; Increasing of pistachio plantation areas in Iran during 2000- 2012

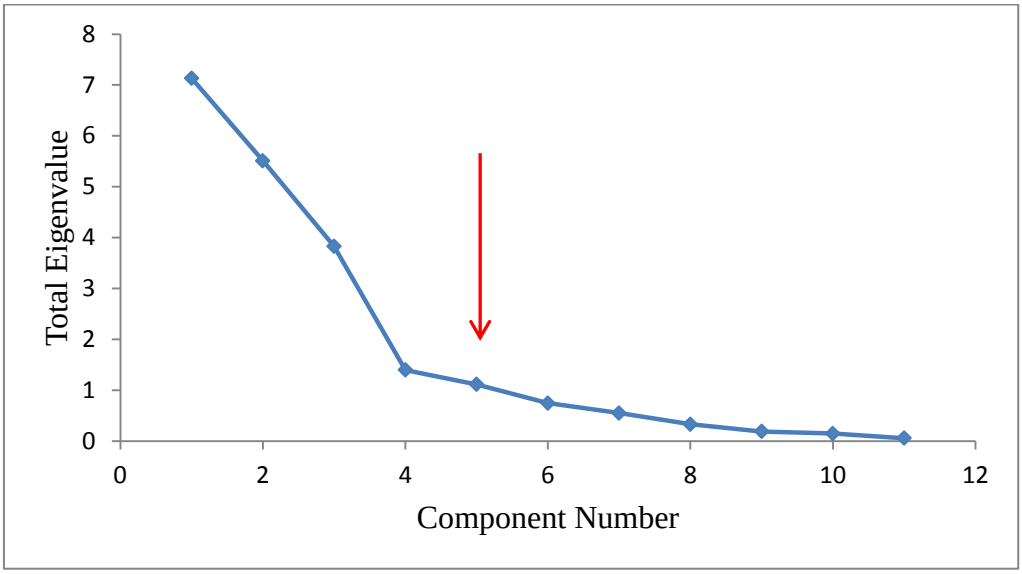


Appendix 2- chapter 1; Pistachio production rates per hectare in Iran during 2000- 2012.

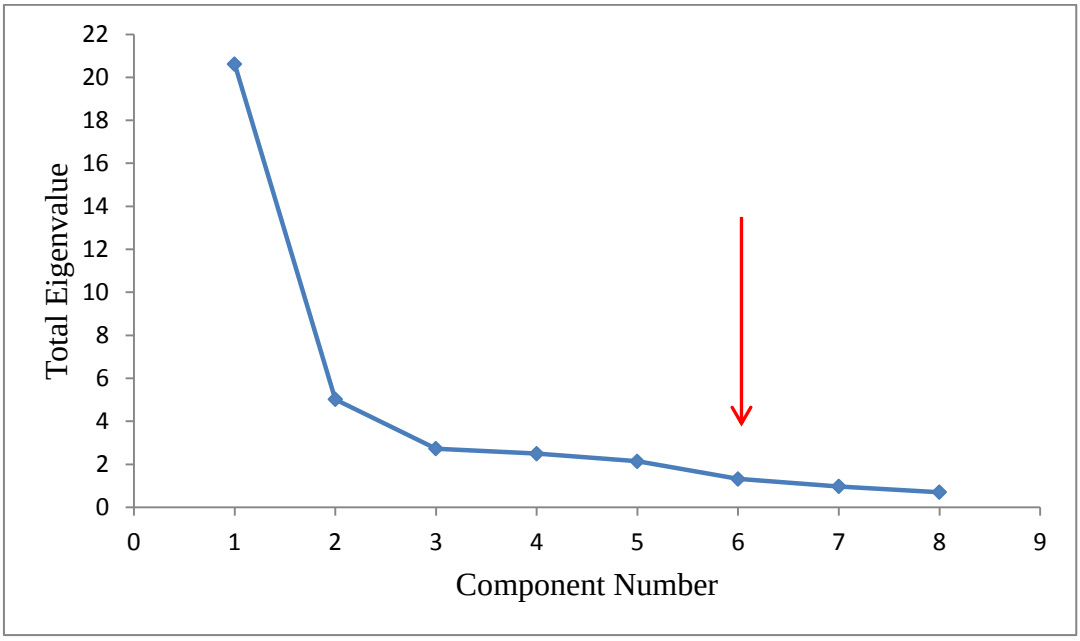
Appendix 3- chapter 3: Composition of Hoagland solution which used in pistachios seedlings experiments (Ranjbar et al. 2002).

Row	Stock name	Elements	Amounts	Dissolve in	Amounts used for 10 distilled water
1	A	KNO_3, KH_2PO_4	100 g 50 g	1 liter distilled water	27.4 ml
2	B	$MgSO_4.7H_2o$, $(NH_4)_2SO_4$	100 g 50 g	1 liter distilled water	27.4 ml
3	C	$Ca(NO3)_2.4H_2o$, EDTA NaFe	400 g 15 g	1 liter distilled water	27.4 ml
4	D	KCl, H_3PO_3, $MnSo_4.H_2o$, $ZnSo_4$, NH_4Mo, $CuSo_4$, H_2So_4,	1 1.1 0.62 g 0.1 g 0.1 g 0.5 g 50 µm	100 ml distilled water	2.74 ml

Appendix 4- chapter 3: Eigenvalues in response to number of components for the estimated parameters of three pistachio rootstocks.



Appendix 5- chapter 5: Eigenvalues in response to number of components for the estimated parameters of three pistachio cultivars.



Appendix 6- chapter 6: Overview of the means values of all measured parameters under severe drought stress treatment (-1.5 MPa) of the drought susceptible, intermediate and tolerant pistachio rootstocks. Parameters that do not have unit, indicated by minus sign (-).

			Pistachio cultivars		
			Badami	**Terebinthus**	**Sarakhs**
Main parameters	**Sub-parameters**	**Unit**	**Drought susceptible**	**Drought intermediate**	**Drought tolerant**
Chlorophyll fluorescence parameters	F_v/F_m	-	0.63 a	0.68 a	0.73 a
	YII	-	0.14 b	0.10 b	0.13 b
	NPQ	-	0.83 a	0.10 a	0.71 a
	qP	-	0.35 b	0.10 b	0.26 b
Growth parameters	PFW	g	22.96 a	31.46 b	18.98 a
	PDW	g	9.9 a	12.82 b	8.86 a
	LDW	g	1.82 b	3.96 b	1.68 a
	RDW	g	4.38 a	6.0 a	4.64 a
	R/S ratio	ratio	4.38 a	0.88 a	1.11 a
	LA	Cm^2	9.52 c	28.12 b	12.38 a
	SEL	Cm	1.1 b	4.14 b	1.56 b
	shedding	g	0.31 a	0.05 a	0.09 a
Nutrient elements	P	%	0.15 b	0.22 a	0.21 b
	K	%	2.06 ab	1.62 a	1.9 a
	Ca	%	1.32 b	2.24 a	1.54 a
	Mg	%	0.2 b	0.6 a	1.54 a
	Fe	ppm	138 a	138 a	100 a
	Zn	ppm	10.72 a	21.96 a	17.56 a
Isotope composition	$\delta^{13}C$	‰	-29.27 a	-28.55 a	-29.81 a

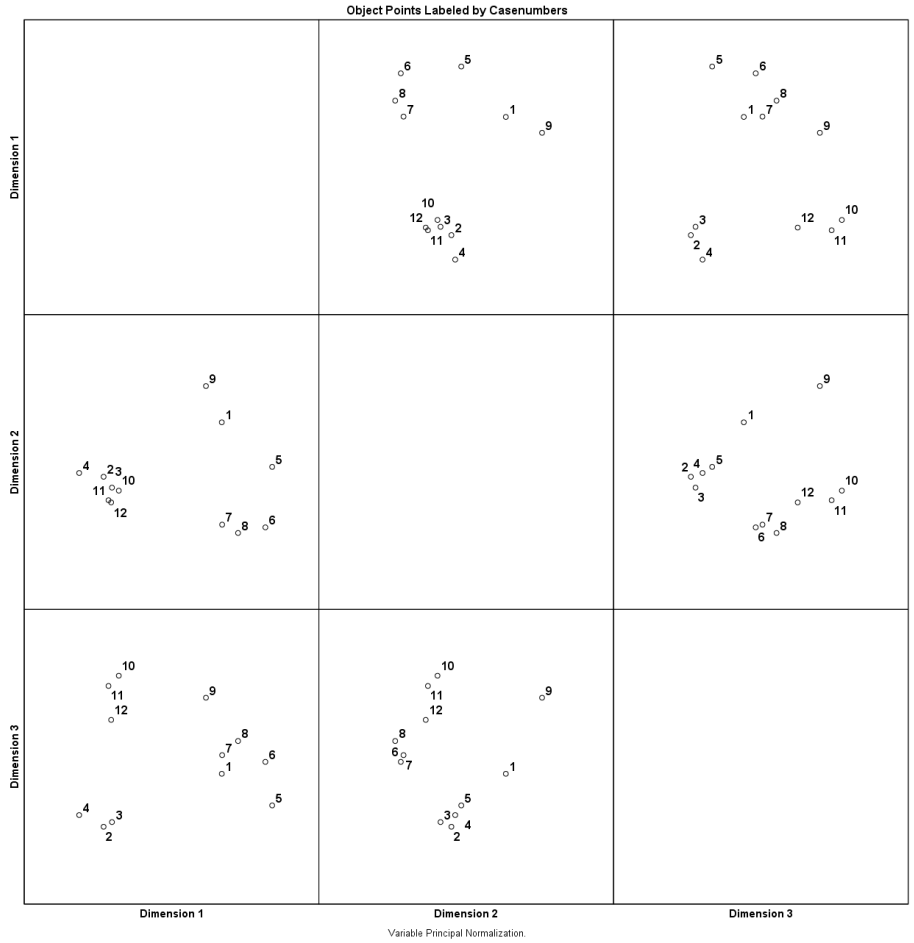
F_v/F_m - maximum quantum yield of PSII
YII- effective quantum yield of PSII electron transport
NPQ- non-photochemical quenching of chlorophyll fluorescence
QP- photochemical quenching of chlorophyll fluorescence
PFW- plant fresh weight
PDW - plant dry weight, biomass
LDW- leaf dry weight
RDW- root dry weight
R/S- root /shoot ratio
LA- leaf area
SEL- stems elongation
P- phosphorous
K- potassium
Ca- calcium
Mg- magnesium
Fe- iron
Zn - zinc
$\delta^{13}C$- carbon isotope composition

Appendix 7- chapter 6: Overview of the mean values of all measured parameters under the severe drought stress treatment (-1.5 MPa) of drought susceptible, intermediate and tolerant pistachio cultivars. Parameters that do not have unit, indicated by minus sign (-).

			Pistachio cultivars		
			Akbari	**Kaleghochi**	**Ohadi**
Main parameters	**Sub-parameters**	**Unit**	**Drought susceptible**	**Drought intermediate**	**Drought tolerant**
Gas exchanges parameters	*Pn*	µmol $m^{-2}s^{-1}$	6 c	7.4 c	9.6 b
	gs	mol H_2O $m^{-2}s^{-1}$	0.07 b	0.06 c	0.09 b
	Ci	µmol CO_2 mol^{-1}	206.9 c	194.4 b	208.4 b
	E	mmol (H_2O) $m^{-2}s^{-1}$	1.3 c	1.4 c	2 b
	WUE	Pn/g_s	110.7 a	115.7 a	107.9 b
Chlorophyll fluorescence parameters	F_v/F_m	-	0.76 a	0.76 a	0.76 a
	YII	-	0.17 a	0.17 a	0.17 a
	NPQ	-	1.41 b	1.78 a	1.94 a
	qP	-	0.39 b	0.44 ab	0.45 a
	ETR		32.7 a	32.7 a	28.8 b
Photosynthesis pigments	Chl *a*	µg/g FW	618.7 b	612.4 b	756 a
	Chl *b*	µg/g FW	254.5 b	247.7 b	282.5 b
	Car	µg/g FW	211 a	209.2 a	259.8 a
Growth parameters	PFW	g	5.9 b	7.5 a	8.8 ab
	PDW	g	2.2 ab	3.1 a	3.5 b
	LDW	g	0.4 b	0.7 a	0.6 b
	RDW	g	1 a	1.2 a	1.5 a
	R/S ratio	ratio	0.8 a	0.65 ab	0.68 a
	SEL	Cm	0.14 b	0.7 b	0.12 b
Nutrient elements	N	% DW	1.46 b	1.4 b	1.36 c
	LWP	MPa	-1.05 c	-1.1 b	-1.13 b
	RWC	%	84.6 a	80.6 b	86.1 a
Osmolyte accumulation	Glucose	g/100 g FW	0.42 a	0.41 a	0.38 a
	Fructose	g/100 g FW	0.54 a	0.5 a	0.37 a
	Sucrose	g/100 g FW	0.99 a	1.03 a	0.9 a
	Starch	g/100 g FW	1.89 a	2.13 a	2.44 a
	Proline	µmol g^{-1} FW	24.8 a	13.8 a	5.4 a
Stomatal	SL	µm	26.7 b	27.9 a	28.1 a

characteristics	SW	µm	16.2 b	17.3 a	17.7 a
	SD	-	290.2 a	306.2 a	247.6 a
Isotope composition	$\delta^{13}C$	‰	-30.09 b	-29.77 a	-29.97 a
	$\delta^{15}N$	‰	-2.71 a	-4.65 a	-3.32 a

Pn- net photosynthesis
g_s- stomatal conductance
C_i- intercellular CO_2
E- transpiration rate
WUE- water use efficiency
F_v/F_m - maximum quantum yield of PSII
YII- effective quantum yield of PSII electron transport
NPQ- non-photochemical quenching of chlorophyll fluorescence
QP- photochemical quenching of chlorophyll fluorescence
ETR- electron transport rate
Chl a- chlorophyll a
Chl b- chlorophyll b
Car- carotenoid
PFW- plant fresh weight
PDW - plant dry weight, biomass
LDW- leaf dry weight
RDW- root dry weight
R/S- root /shoot ratio
SEL- stems elongation
N- nitrogen
LWP- leaf water potential
RWC- relative water content
SL- stomata length
SW- stomata width
SN- stomatal number
$\delta^{13}C$- carbon isotope composition
$\delta^{15}N$- nitrogen isotope composition



Appendix 8- chapter 3: The results from drawing PC1 on PC2 and PC1 on PC3 showed that there are not major difference between cultivars and rootstocks on biplot graph under water stress conditions. The PC3 increased the dispersion of data to PC2 on biplot space. PC3 explain less variation than PC2 and has lower reliability.