EL SABER DE MIS HIJOS
HARA MI GRANDEZA

UNIVERSIDAD DE SONORA

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD

Departamento de Investigación y Posgrado en Alimentos

Programa de Posgrado en Ciencias y Tecnología de los Alimentos

Desarrollo de estrategias de riego deficitario controlado y selección del portainjerto para mejorar la calidad y funcionalidad de los pistachos (*Pistacia vera)*

TESIS

Como requisito parcial para obtener del grado de:

DOCTOR EN CIENCIAS DE LOS ALIMENTOS

Presenta:

M. en C. Luis Noguera Artiaga

Hermosillo, Sonora Noviembre de 2019

APROBACIÓN

Desarrollo de estrategias de riego deficitario controlado y selección del portainjerto para mejorar la calidad y funcionalidad de los pistachos (*Pistacia vera*)

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AGRADECIMIENTOS

A mis directores, Ángel y Armando, por su apoyo incondicional durante toda esta tesis. Con la confianza depositada en mí en estos años se han ganado un amigo (no sé si será bueno o malo para ellos, pero es lo que hay ;-) Las experiencias vividas con vosotros son completamente inefables (bateando para primera ¡eh!).

Al grupo de CSA, porque sí, porque sin amigos no se puede trabajar y ahí hay unos cuantos.

A Paco Burló, Esther Sendra, Paqui Hernández y David López por sus consejos, por su cariño, por todo lo que me han enseñado en estos años. Gracias.

Al Dr. Hussein Ali Sulayman El Zaeddi y a Jesús Clemente por hacerme mejor persona.

A Pedro Zapata por ser Pedro Zapata. Espero poder recompensarte algún día todo lo que has hecho por mí.

Al Departamento de Tecnología Agroalimentaria. A todos.

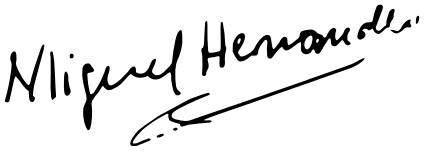
Al Departamento de Investigación y Posgrado en Alimentos de la Universidad de Sonora por acogerme como uno más y brindarme todo su cariño. A Carina, Javier y Reyna Luz por sus consejos y recomendaciones. Y, en especial, quiero agradecer a Carmen, Violeta, y Mario los buenos momentos que me hicieron vivir. Con vosotros, nunca me sentí fuera de casa.

A Joel por todo su apoyo. Porque fue un pilar fundamental en esta tesis, tanto dentro como fuera del DIPA y porque será mi hermanito para siempre.

Al *ola k ase*, porque soy lo que soy en buena parte gracias a vosotros. Porque el mundo está lleno de cabrones y tener a unos cerca siempre ayuda.

A mis padres,
a mi hermano Eduardo,
a Paola.
Sin vosotros, nada valdría la pena.

Si me muero, que me muera
con la cabeza muy alta.
Muerto y veinte veces muerto,
la boca contra la grama,
tendré apretados los dientes
y decidida la barba.
Cantando espero a la muerte,
que hay ruiseñores que cantan
encima de los fusiles
y en medio de las batallas.



CONTENIDO

INTRODUCCIÓN

Hay constancia de que los pistachos han formado parte de la alimentación del ser humano desde, al menos, finales del paleolítico. El pistacho es el fruto del pistachero, árbol del género *Pistacia*. Es una planta desértica con gran tolerancia al suelo salino. Es un árbol caducifolio, similar al almendro, que puede llegar a alcanzar los 10 m de altura, con flores masculinas y femeninas separadas en árboles diferentes. Es una especie muy longeva (puede llegar a vivir entre 150 y 300 años), su desarrollo es muy lento y es bastante resistente a condiciones climáticas extremas, pudiendo sobrevivir en temperaturas que van desde -10 ºC en invierno, hasta 40 ºC en verano. Los árboles se plantan en huertos y necesitan de 7 a 10 años para lograr una producción considerable. La producción es alterna, o bienal, lo cual significa que la cosecha es más abundante cada dos años. La producción pico se alcanza aproximadamente a los 20 años.

El pistachero es una especie xerófita que requiere temperaturas moderadamente bajas en invierno. Para que el árbol tenga una óptima y homogénea brotación es preciso la acumulación durante el reposo invernal de un determinado número de horas frío, variable según los cultivares considerados. No tiene gran capacidad de enraizamiento por lo que es preciso el uso de portainjertos para su propagación vegetativa. Los estudios en portainjertos de pistachero son poco numerosos y, en general, sólo se han centrado en comparar la respuesta productiva y/o la resistencia a enfermedades. La elección del portainjerto es una de las decisiones más importantes para el desarrollo de la plantación y es diferente según las zonas de cultivo. Tradicionalmente se ha considerado el pistachero como un árbol muy resistente a la sequía y la salinidad, aunque la respuesta al estrés hídrico desde el punto de vista de la calidad de los frutos obtenidos ha sido muy poco caracterizada.

El pistacho es el sexto fruto seco en superficie a nivel mundial después del anacardo, almendro, nogal, avellano y castaño. La producción mundial se ha incrementado enormemente, llegando incluso hasta casi triplicarse en los últimos 20 años.

La tierra tiene tres cuartas partes cubiertas de agua, pero solo un 2.5 % es de agua dulce y la mayor parte de esta se encuentra congelada en el hielo de Groenlandia y la Antártida, es subterránea o está contaminada, por lo que solo disponemos de un 1 % para el consumo humano, la agricultura y la industria. Este es un problema que se agrava con el cambio climático, el aumento de la población, el crecimiento de las ciudades, un alto nivel de consumo y una mala gestión del agua.

La sequía afecta ya al 17 % del territorio europeo, causando pérdidas de más de 100 billones de euros en los últimos 5 años. Una de las áreas en la que más se ha enfocado esta problemática es la agricultura. Esta se enfrentará, en un futuro muy cercano, a restricciones hídricas que se aplicarán durante todo el año, permitiendo el riego únicamente en momentos concretos, con el fin de llevar a cabo un buen reparto del agua disponible. Por ello, es necesario conocer a fondo las necesidades hídricas de los cultivos, caracterizándolos de acuerdo a su demanda de agua y a su resistencia al estrés hídrico.

A finales de la década de los 80, comenzaron a diseñarse programaciones de riego deficitario controlado (RDC) en pistachero. Estas técnicas se aplican a especies leñosas y se basan en la distinta sensibilidad de las diferentes fases fenológicas del cultivo al estrés hídrico. A día de hoy, se han llevado a cabo multitud de caracterizaciones del cultivo estudiando la respuesta al estrés hídrico aplicado mediante RDC, aunque la inmensa mayoría de estas se han centrado en la respuesta fisiológica del cultivo y en la producción obtenida, dejando de lado la calidad de los frutos obtenidos.

JUSTIFICACIÓN

La necesidad de optimizar el uso del agua en los agrosistemas es un hecho asumido por agricultores y organismos reguladores. Esta situación irreversible nos obliga a convivir con la escasez de recursos hídricos y a desarrollar herramientas capaces de asegurar la competitividad de la fruticultura. Sin embargo, la mayoría de las estrategias de riego deficitario no están basadas en niveles de déficit hídrico concretos en las plantas, ni está claro que conlleven una mejora de las características funcionales y sensoriales de los frutos. En este sentido, la bibliografía existente es escasa y contradictoria, debido fundamentalmente a que: (i) en ciertas publicaciones falta precisión a la hora de definir el estado hídrico del cultivo estudiado (duración del estrés, velocidad de imposición del mismo, etc.) y (ii) se presupone la existencia de una relación directa entre déficit hídrico y acumulación de compuestos bioactivos.

Debido a la situación anteriormente expuesta, en la presente investigación se aplicaron dos tratamientos de riego deficitario controlado y se emplearon tres portainjertos durante el cultivo del pistacho, con el fin de estudiar la incidencia que cada uno de ellos tiene sobre la calidad de los frutos obtenidos.

HIPÓTESIS DE PARTIDA

La hipótesis principal de esta Tesis Doctoral radica en que, no hay relación directa entre el nivel de compuestos bioactivos de los frutos y el nivel de estrés hídrico (controlado); mientras que bajo un estrés hídrico elevado se produce una importante regulación estomática y el CO_2 se destina al mantenimiento del metabolismo primario; bajo un déficit moderado/suave se redistribuye el CO_2 hacia la formación de metabolitos secundarios en detrimento del crecimiento. Esto da lugar a frutos con mayor contenido en compuestos bioactivos en aquellos frutos en los que el déficit hídrico ha sido controlado. Además, las situaciones de estrés hídrico provocan una acumulación de sustancias antioxidantes, como respuesta fisiológica a la eliminación de radicales libres formados. Sin embargo, para una misma especie, el incremento de la actividad antioxidante o acumulación de sustancias del metabolismo secundario no siempre parece seguir unas pautas fáciles de describir o relacionar con el estado hídrico; en la mayor parte de los casos la falta de correlación se debe a que el estado hídrico no es completamente comparable por una inadecuada o insuficiente descripción.

Por ello, pensamos que mediante el manejo preciso de niveles de déficit hídrico "suave" es factible conseguir mínimas mermas en la producción y generar a su vez productos de máxima calidad. Estos productos, por ser el resultado de una optimización del uso del agua y tener una calidad elevada, tendrán una identidad marcada que los diferenciará del resto de su categoría, facilitando su inclusión en mercados internacionales más exigentes.

Este hecho supondría un incuestionable avance para la fruticultura, ya que se podrían obtener productos con un claro valor añadido al percibirse como productos sostenibles y respetuosos con el medioambiente.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

Para probar la hipótesis planteada, el trabajo experimental se dividió en cinco etapas, las cuales se describen en los siguientes cinco capítulos:

Capítulo 1

Atributos de calidad de diferentes cultivares de pistacho

Este capítulo consiste en un artículo científico titulado "Functional and sensory properties of pistachio nuts as affected by cultivar", publicado en la revista *Journal of the Science of Food and Agriculture*. En él se refleja la información obtenida tras estudiar las propiedades físico-químicas, funcionales y sensoriales de ocho cultivares de pistacho.

Capítulo 2

Concepto de hidrosostenibilidad

Este capítulo está compuesto por un artículo científico titulado "Opinion of Spanish consumers on hydrosustainable pistachios", publicado en la revista *Journal of Food Science.* El artículo contiene información sobre el desarrollo del concepto hidroSOStenible o hidroSOS obtenida mediante el empleo de grupos focales, con el fin de obtener información que permita a los productores obtener un producto con una calidad diferenciada.

Capítulo 3

Calidad físico química y sensorial de pistachos hidrosostenibles

Este capítulo está formado por dos artículos científicos. El primero de ellos se titula "Influence of regulated deficit irrigation and rootstock on the functional, nutritional and sensory quality of pistachio nuts" y está aceptado para su publicación en la revista *Scientia Horticulturae*, mientras que el segundo, está titulado "Influence of rootstock and regulated deficit irrigation on pistachio quality" y está enviado para su publicación a la revista *Foods*. En ambos se estudia el efecto que el empleo de diferentes tratamientos de riego deficitario controlado y portainjertos tienen sobre la calidad físico-química, funcional y sensorial de los pistachos. Esta última, se estudió mediante el empleo de análisis sensorial descriptivo mediante panel entrenado y un estudio afectivo desarrollado en España, México y Polonia.

Capítulo 4

Estudio de la calidad funcional de pistachos hidrosostenibles

Este capítulo consiste en un artículo científico titulado "Phenolic and triterpenoid composition and inhibition of α-amylase of pistachio kernels (*Pistacia vera* l.) as affected by rootstock and irrigation treatment", publicado en la revista *Food Chemistry.* En él se estudió la influencia que el riego deficitario controlado y el empleo de diferentes portainjertos tuvo sobre el contenido polifenólico, contenido de triterpenoides, capacidad antioxidante y capacidad de inhibición de alfa-amilasa de los pistachos obtenidos.

Capítulo 5

Estudio de las propiedades antimutagénicas y citoprotectivas

Este capítulo, contiene información sobre la incidencia que el riego deficitario controlado y el empleo de diferentes portainjertos tienen sobre las propiedades antimutagénicas y citoprotectivas de los frutos de pistacho. Está formado por una publicación titulada "Effect of rootstock and regulated deficit irrigation on antioxidant, antimutagenic, and cytoprotective properties of pistachios" enviada para su publicación a la revista *Molecules*.

Capítulo 1

Atributos de calidad de diferentes cultivares de pistacho

PUBLICATION 1

FUNCTIONAL AND SENSORY PROPERTIES OF PISTACHIO NUTS AS AFFECTED BY CULTIVAR

Luis Noguera-Artiaga, María Desamparados Salvador, Giuseppe Fregapane, Jacinta Collado-González, Aneta Wojdyło, David López-Lluch, and Ángel A. Carbonell-Barrachina

Journal of the Science of Food and Agriculture, 99, 6696-6705, 2019.

DOI: https://doi.org/10.1002/jsfa.9951

Índice de calidad

Journal Citation Reports (2018)

Área: Agriculture multidisciplinary

Factor de impacto: 2.422 (9/57)

Cuartil: Q1

FUNCTIONAL AND SENSORY PROPERTIES OF PISTACHIO NUTS AS AFFECTED BY CULTIVAR

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ABSTRACT

BACKGROUND: Modern agriculture allows farmers to choose among different cultivars of the same fruit to fulfill their agronomic needs and consumers' demands; however, there are only few studies describing and comparing key functional and sensory properties of different pistachio cultivars. The main objective of this study was to compare eight pistachio cultivars by analyzing key functional properties (phenolic compounds, polymeric procyanidins, antioxidant activity, inhibition of α-amylase and β-glucosidase), aromatic compounds (GCMS), and sensory properties with a trained panel.

RESULTS: By combination of LC-PDA-MS-QTof and electrospray ionization were determinate 2 phenolic acids, 9 flavonols, 1 anthocyanin and 3 flavan-3-ols in pistachio cultivars, with a total concentration ranged from 500 to 6065 mg 100 g^{-1} dry weight (dw); total polymeric procyanidins concentrations oscillated between 348 and 5919 mg 100 g^{-1} dw, being (-)-epicatechin the major monomer contributor; Pinene was the most abundant volatile compound (~200 mg kg^{-1} dw); and, in case of sensory analysis of samples, 23 sensory attributes were found significantly different.

CONCLUSION: The cultivar "Larnaka" stood out as having the best functional profile (high polyphenolic content, high antioxidant activity, and high values of α-amylase and β-glucosidase inhibition), while the cultivars "Kastel" and "Kerman" showed the most attractive sensory properties, mainly the most intense flavor.

Keywords: *Pistacia vera* L. cultivars; GC-MS; antioxidant activity; phenolic compounds; LC-PDA-MS-QTof.

1. INTRODUCTION

The pistachio (*Pistacia vera* L.) belongs to the *Anacardiaceae* family. This nut is originated in western Asia and Asia Minor, where it can still be found growing wild. Nowadays, it is commercially cultivated in countries such as Iran, Turkey, United States, Syria, Greece, Italy, and Spain. The Islamic Republic of Iran is the first world pistachio producer with a production of 241,759 tons, whereas Spain is the tenth producer with 2,500 tons (Food and Agriculture Organization of the United Nations, 2015).

The pistachio tree has been cultivated for a long time in semi-arid areas and it is considered as a desert-tolerant plant mainly for its ability to survive under extreme water stress conditions.(Memmi, Gijón, Couceiro, & Pérez-López, 2016) The importance of pistachio is due to its fruit, which is formed by the shell and the edible kernel that has a thin skin and light green flesh with a distinctive and attractive flavor (Dreher, 2012; Saitta, La Torre, Potortì, Di Bella, & Dugo, 2014).

Pistachio has been used as a folk remedy since prehistoric times due to its nutritional value and long storage life. Recently, it has been demonstrated that consumption of pistachio significantly decreases the oxidative stress and improves both total cholesterol and LDL levels. Indeed, the United States Food and Drug Administration approved the first qualified health claim (July 2003) specific to seeds and the risk of heart disease, quoting that "*scientific evidence suggests but does not prove that eating 1.5 ounces (42.5 g) per day of most nuts, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease*". In addition, its antioxidant capacity and anti-inflammatory potential have been also reported, and was basically linked to its high total phenolic content.(Alasalvar & Bolling, 2015; Mandalari et al., 2013) Therefore, nowadays, pistachio kernels are consumed roasted and salted as snacks, and they are also incorporated into a wide array of food products such ice cream, salads, fermented meats and bakery products (Dreher, 2012; Saitta et al., 2014).

Even though the chemical composition of the pistachio seeds is complex and is still not completely known, some studies have reported that among its micro-constituents the following are the most relevant, unsaturated fatty acids [both MUFAs (monounsaturated fatty acids) and PUFAs (polyunsaturated fatty acids)], proteins, dietary fiber, vitamin K, and phytochemicals, such as phytosterols, lutein (xanthophyll carotenoid), γ-tocopherol, and some polyphenols. In addition, they are considered an excellent source of K, P, Mg, and Ca.(Fabani et al., 2013) All these components can act synergistically helping to promote cardiovascular health, glycemic and weight control, and promoting protective effect against colorectal and breast cancer (Dreher, 2012; Saitta et al., 2014). Some studies have stated that the

chemical composition of pistachio oil depends on environmental factors such as climate, geographical origin and soil type, among others, and some intrinsic factors such as variety (Chahed et al., 2008).

The aim of this work was to study the functional, aromatic, and sensory characteristics of eight pistachio cultivars to fully characterize them and to gather as much information as possible to help farmers and consumers to take proper decisions when growing and buying pistachios and pistachio-based products, in particular regarding their healthy and organoleptic properties.

2. MATERIAL AND METHODS

2.1. Chemicals

Standards including 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tri(2-pyridyl) striazine (TPTZ), potassium persulfate, acetic acid, phloroglucinol, and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). (+)-Catechin, and (−)-epicatechin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, procyanidin B1 and B2, eriodictyol-3-*O*-glucoside, quercetin and keampferol-3-*O*-glucoside were obtained from Extrasynthese (Lyon Nord, France). Ascorbic acid and acetonitrile for ultra-pressure liquid chromatography (UPLC, gradient grade) were obtained from Merck (Darmstadt, Germany).

2.2. Plant material and experimental design

The experiment was carried out on an experimental farm *"El Chaparrillo",* Ciudad Real, Spain (39◦ 00' N, 3◦ 56' W, altitude 640 m) in crop season 2014-15. The plant material consisted of 4 pistachio trees of eight cultivars (cvs.) Kerman, Avdat, Larnaka, Mateur, Napolitana, Aegina, Kastel and Sirora (Figure 1). Tree spacing followed a 7 × 6 m pattern (238 trees ha^{-1}). The climate of the area had an average annual rainfall of 397 mm, mostly distributed outside a four-month summer drought period, and soil is a shallow clay-loam (*Petrocalcic Palexeralfs*) of 0.5 m depth, and a discontinuous *petrocalcic* horizon of around 0.5 m. All the pistachio trees were subjected to the same farming/ agronomical practices (irrigation, fertilization, pruning, and harvesting).

2.3. Polyphenols extraction

Polyphenols from grounded samples were extracted following the procedure previously described by Noguera-Artiaga, Pérez-López, Burgos-Hernández, Wojdyło, and Carbonell-Barrachina (2018). After extraction, samples were filtrated through a

0.20 µm membrane filter (Millex Samplicity® Filters Membrane) immediately before analysis.

2.4. Determination of polyphenols by LC-PDA-MS-QTof

Chromatographic analyses were carried out on a UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm, Waters Corp.; Milford, USA) at 30 °C. The mobile phase used consisted of two solvents: water-formic acid (2.5 % formic acid, *v/v*) as solvent A, and acetonitrile pure as solvent B. The injection volume was 10 µL and elution was performed at a flow rate of 0.45 mL min^{-1}. The linear gradient started maintaining from 0 to 1 minute the 99 % A, reaching the 0 % A in the minute 12 and from 12.5 to 13.5 min the initial composition (99 % A) was used for re-equilibrating the column. Identification of the compounds was made using an Acquity UPLC system (Waters, Milford, MA), and a Micromass Q-Tof spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative mode. The eluent was passed to the electrospray source with the following conditions: capillary voltage of 2500 V, cone voltage of 30 V, source temperature of 130 °C, desolvation temperature of 350 °C and desolvation gas (nitrogen) flow rate 300 L h^{-1}. The full scan mass covered the range from 100 to 1500 *m/z*. For identification of polyphenols, spectral data from all peaks were accumulated at 280 nm (flavan-3-ols), 320 nm (phenolic acids), 360 nm (flavonols and flavanones), and 520 nm (anthocyanins). All Q/TOF-MS chromatograms are presented as the base peak intensity (BPI) chromatograms and scaled to 12.400 counts per second, cps (=100 %). Injection of standard solutions of known concentrations was carried out for quantification of polyphenols, according to Wojdyło, Nowicka, Carbonell-Barrachina, and Hernández (2016). Results were expressed as mg *per* 100 g dry weight (dw) and all data were obtained in triplicate. The leucine encephalin was used as internal reference compound and the lock mass correction was ±1.000 for the mass window.

2.5. Polymeric procyanidins

Pistachio samples (0.05 g) were treated with a solution of HCl/MeOH/phloroglucinol/ascorbic acid [0.8 mL of the methanolic solution of phloroglucinol (75 g/L) and ascorbic acid (15 g/L)]. After 30 min, an aqueous sodium acetate solution (1.2 mL) was added to stop the reaction of phloroglucinol at 4 ºC (Wojdyło et al., 2016). Phloroglucinol adducts were analyzed following the methodology previously described by Noguera-Artiaga et al. (2018). All determinations were performed in triplicate and expressed as mg *per* 100 g dw.

2.6. Analysis of antioxidant activity

The solvent prepared for analysis of antioxidant activity was prepared following the method previously described by Wojdyło, Jáuregui, Carbonell-Barrachina, Oszmiański, and Golis (2013). The free radical scavenging capacities were determined using the $ABTS^+$ method described by Re et al. (1999) and FRAP (ferric reducing antioxidant power) method described by Benzie and Strain (1996). Determinations of $ABTS^+$ and FRAP methods were performed using a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). All antioxidant capacity analyses were done in triplicate, and results were expressed as mmol Trolox *per* 100 g dw.

2.7. α-Amylase and α-glucosidase inhibition

The α-amylase and α-glucosidase inhibition were assayed according to the procedure described by Nowicka, Wojdyło, and Samoticha (2016). The α-amylase inhibitory effect was assayed by the reducing groups released from starch by the reduction of 3,5-dinitrosalicylic acid (DNS), while in the case of the inhibition of α-glucosidase activity, the determination was carried out by measuring the amount of glucose hydrolyzed from *p*-nitrophenyl-α-D-glucopyranoside. Then, the absorbance was measured at 540 and 405 nm (for α-amylase and α-glucosidase, respectively) using a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). The analysis was run in triplicate and results were expressed as IC_{50} (mg of dried nut mL^{-1}).

2.8. Extraction of volatile aroma compounds

The extraction of the volatile compounds of the pistachios fruits was carried out using the headspace solid-phase micro-extraction method (HS-SPME). Five grams finely ground pistachios sample was mixed with 1 g of NaCl, stirred with a magnetic bar (500 rpm) and hermetically placed in 50 mL vials with polypropylene caps and PTFE/silicone septa. Vials were equilibrated for 10 min at 40 °C (to simulate mouth temperature during the chewing process) and, after this equilibration time, a 50/30 μm DVB/CAR/PDMS fiber (length of 2 cm) was exposed to the sample headspace for 50 min at 40 °C. Volatile extractions were run in triplicate.

2.9. Chromatographic analysis of volatile compounds by GC-MS

The isolation and identification of the volatile compounds were performed on a gas chromatograph, Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detect or GCMS QP-5050A. The GC–MS system was equipped with a Restek Rxi-1301 Sil MS column (30 m × 0.25 mm, 1 μm film thickness; Restek Corporation, Bellefonte, PA, U.S.A.). Helium was used as carrier gas at a flow rate of 0.6 mL min^{-1} in a splitless mode. The oven temperature started at 80 °C and was increased by 3 °C min^{-1} up to 210 °C; after 1 min of

stabilization the temperature was increased by 25 °C min^{-1} up to 300 °C, and it were hold for 5 min. Detector and injector temperatures were 300 and 230 °C, respectively. Desorption time of the samples was 3 min (230 °C).

Most of the volatile compounds were identified using different analytical methods: (1) retention indices [Kovat's index, where retention time (RT) of each peak is normalized to the RT of adjacently eluting n-alkanes (alkane standard C8-C20)], (2) GCMS retention indices (authentic chemicals), and (3) mass spectra (NIST05 spectral library collection). Identification was considered tentative when it was based on only mass spectral data. Results were expressed as a relative concentration (mg kg^{-1}) by each one of the volatile compounds.

2.10. Sensory analysis

Twelve highly trained panelists (7 female and 5 male), with more than 500 h of testing experience with nuts, aged between 24 and 61 years, and associated with the AgroFood Department of the Universidad Miguel Hernández de Elche (Orihuela, Alicante, Spain), participated in this study. For the analysis, 20 fruits for each panelist were served in coded disposable covered plastic plates, in a tasting room at room temperature (22±2 ºC) with an illumination of fluorescent light. Six 1 h-sessions were held for the evaluation of 8 samples by triplicate (4 samples in each session).

Thirteen appearance, 6 flavor and 6 texture descriptive attributes were used for the evaluation of the samples. For the quantification of the intensity of samples attributes, the panel used a numerical scale from 0 to 10, with increments of 0.5 units, where 10 represents extremely strong and 0 none intensity.

2.11. Statistical analysis

The data was subjected to two-way analysis of variance (ANOVA) and later to Tukey's multiple-range test to compare the means. Differences were considered statistically significant at $p<0.05$. All statistical analyses were done using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD).

3. RESULTS AND DISCUSSION

3.1. Characterization of Phenolic compounds.

It is important to emphasize the fact that polyphenols present antioxidant and anti-inflammatory properties (Duthie, Gardner, & Kyle, 2003; C Gentile et al., 2012). Polyphenols represent the main dietary antioxidant; it has been demonstrated that these compounds have higher antioxidant capacity *in vitro* compared with other

bioactive compounds such as vitamins and carotenoids (Mandalari et al., 2013; Richelle, Tavazzi, & Offord, 2001) .

The combination of LC-PDA-MS-QTof analysis and electrospray ionization (ESI) mass spectrometry used revealed the presence of a wide range of polyphenols in the analyzed pistachio nuts samples: 2 phenolic acids, 9 flavonols, 1 anthocyanin and 3 flavan-3-ols (as dimer of procyanidins and (-)-epicatechin and (-)-epicatechin-*O*-hexoside) (Table 1). These phenolic compounds were identified according to their retention times, molecular mass, fragmentation patterns, characteristic spectra, and by using some literature references (Fabani et al., 2013; Grace et al., 2016; Gültekin-Özgüven, Davarcı, Paslı, Demir, & Özçelik, 2015; Regueiro et al., 2014).

Previously, other authors (Saitta et al., 2014; Tomaino et al., 2010) identified in pistachio samples hydroxybenzoic acids (as gallic and protocatechuic acids) and hydroxycinamic acid (as chlorogenic acid), flavan-3-ols (as [+]-catechin and [-]-epicatechin), flavonols (as glycosides of quercetin), flavones (as luteolin), isoflavones (as daidzein), and flavanones (as aglycones of eriodictyol and their glycosides), being gallic acid, (+)-catechin, and isoquercetin the major compounds identified.

The content of phenolic compounds in different pistachio samples ranged from 500 mg 100 g^{-1} dw of samples of the "Sirora" cultivar to 6065 mg 100 g^{-1} dw of "Larnaka" cultivar samples (Table 2). The concentration of phenolic compounds found in this work was similar to those reported in pistachio cultivated in other countries, such as Turkey (461 mg GAE 100 g^{-1} dw), United States of America (~400 mg GAE 100 g^{-1} dw), Italy (178.57 mg GAE 100 g^{-1} dw), Austria (492 mg of GAE 100 g^{-1} dw), and Greece (1442 mg of GAE 100 g^{-1} dw) (Ballistreri, Arena, & Fallico, 2009; C Gentile et al., 2012; Kornsteiner, Wagner, & Elmadfa, 2006; Wu et al., 2004; Yang, Liu, & Halim, 2009). Besides, Tsantili, Konstantinidis, Christopoulos, and Roussos (2011) indicated that differences between studies on the same cultivar could be ascribed to field and/or measuring conditions and postharvest handling before sampling.

Pistachios are the only nuts that contain anthocyanins, which are the pigments of the skin responsible for the colors of many vegetables and fruits.(Tomaino et al., 2010) The most abundant compound of this family (cyanidin-3-*O*-galactoside) in the studied cultivars ranged between 12.3 and 130.6 mg 100 g^{-1} dw, with samples of the cultivars "Kastel" and "Avdat" having the lowest and highest anthocyanin content, respectively.

All pistachio cultivars studied here had higher anthocyanin content than those previously analyzed by Ballistreri et al. (2009) and Seeram et al. (2006), who studied the effect of ripeness and drying or roasting process on anthocyanin content in Californian and Italian (var. *Bianca*) pistachios. The "Aegina", "Mateur" and "Sirora" samples had similar cyanidin-3-*O*-galactoside contents than that found by Bellomo

and Fallico (2007) in pistachio samples of different geographic origin and at different ripening stage. Cultivars "Larnaka" and "Napolitana" had similar content of anthocyanin than observed for cyanidin-3-*O*-glucoside by Tomaino et al. (2010) in var. *Bronte,* and for cyanidin-3-*O*-galactoside by Bellomo and Fallico (2007) in var. *Agrigento.* All these findings indicated that anthocyanin content may be influenced by several factors including cultivar, geographical origin, ripening stage and industrial processing.

3.2. Characterization of polymeric procyanidins

By comparing their retention times and UV and mass spectral properties with those of pure standards, 3 flavan-3-ols and 4 polymeric procyanidins were identified in pistachio seeds [(-)-epicatechin, (-)-epicatechin-O-hexoside, and dimer of procyanidins], and were evaluated as polymeric procyanidins after phloroglucynolysis reaction. The total polymeric procyanidins concentrations oscillated between 348 and 5919 mg 100 g^{-1} dw, with (-)-epicatechin units being the major monomer contributor to these compounds (Table 2); this wide range indicated the huge variability in the content of proanthocyanidins in pistachio nuts. The highest polymeric procyanidins concentration was found in samples of the "Larnaka" cultivar (5919 mg 100 g^{-1} dw), whereas the lowest ones (from 348 to 506 mg 100 g^{-1} dw) were found in the cultivars "Aegina", "Avdat" and "Sirora", respectively. Fabani et al. (2013) studied the (+)-catechin and epicatechin contents in pistachios cultivated in Argentina *(Pistacia vera*, variety "Kerman") showing that these compounds presented a content of 16 and 3 μg g^{-1} dw, respectively. Also, C. Gentile et al. (2007) reported that pistachios (*Pistacia vera* L., variety "Bronte") cultivated at the region of Sicilia (Italy) had a content of 2680 μg g^{-1} of proanthocyanidins including not only monomers. These data indicate that "Larnaka" (5919 μg g^{-1} dw), "Kastel" (4600 μg g^{-1} dw), and "Napolitana" (3825 μg g^{-1} dw) cultivars were richer in procyanidins than those reported in the Sicilian varieties. In addition, Gu et al. (2003) reported that pistachios, in general, had a procyanidin content of 109.9 μg g^{-1} indicating that had the second highest content of these chemical group in the nuts family after pecans. However, data from the current study indicated that pistachios grown in Spain would occupy the first place in terms of the content of flavan-3-ols monomers.

3.3. Antioxidant activity

Plants, in general, are rich in secondary metabolites, which play an important role in scavenging free radicals. This fact not only allows these plants to be considered as a source of multifunctional properties, but they can be seen as possible sources for new drugs aimed at various types of diseases (Chadha, Engle, Hughes, Ledesma, & Weinberger, 2011; Dastmalchi, Dorman, Vuorela, & Hiltunen, 2007). Due

to the complex nature of those phytochemicals, it is necessary to use more than one method to evaluate antioxidant capacity of plant materials. For this reason, in this study the antioxidant activity of the different pistachio cultivars was evaluated by different chemical assays: $ABTS^{+}$ and the ferric-reducing antioxidant power (FRAP) assays. Generally, good correlation is found between both methods, as they use the same single electron transfer mechanism. $ABTS^{+}$ method is based on the ability of antioxidant to reduce the $ABTS^{+\bullet}$ radical and is one of the most applied method for its high sensitivity, practicality, speed and stability. In addition, this method allows to confirm the antiradical capacity of the hydrophilic and lipophilic antioxidants, because it can be used in both organic and aqueous solvent systems in comparison with other tests with antioxidants (Rakholiya, Kaneria, & Chanda, 2017). The free radical scavenging capacities of $ABTS^{+\bullet}$ showed that "Kerman" (6.21 mmol Trolox 100 g^{-1} dw) and "Larnaka" (5.88 mmol Trolox 100 g^{-1} dw) and "Napolitana" cultivars presented the highest antioxidant capacity values (Table 3); whereas the lowest were found for "Mateur" and "Sirora" (0.40 and 0.46 mmol Trolox 100 g^{-1} dw respectively). The antioxidant activity of the hydrophilic fraction of pistachios was also measured by the FRAP method. This method is based on the ability of an antioxidant to reduce Fe^{+3} in the presence of 2,4,6-Tripyridyl-s-triazine (TPTZ), forming a Fe^{+2}-TPTZ complex (Kaneria, 2017). Results obtained by FRAP method were comparable to those from $ABTS^{+}$, although FRAP method exhibited that not only "Larnaka" (3.61 mmol Trolox 100 g^{-1} dw) and "Kerman" (3.19 mmol Trolox 100 g^{-1} dw) presented the highest antioxidant capacity, but also samples of the cultivars "Napolitana" (3.61 mmol Trolox 100 g^{-1} dw) and "Avdat" (3.19 mmol Trolox 100 g^{-1} dw) showed high values of this key functional parameter (Table 3). Once again "Sirora" showed the lowest FRAP antioxidant activity.

These results showed that the total content of phenolic compounds was not directly proportional to antioxidant activity, except in the case of the cultivar "Sirora", which had the lowest content in both parameters. In this sense, $ABTS^{+}$ and FRAP assays data showed that type of phenolics (composition) rather than the amounts is responsible for high antioxidant activity. This can be contrasted by the amount of gallic acid found in the samples. Cultivars "Larnaka" and "Kerman" had high contents of this compound and were in turn the ones with the best antioxidant behavior in the two methods studied. This finding agreed with other authors who reported that differences in antioxidant activities of plant extracts could be due to different structures of plant extracts from phenolic acids and flavonoids compounds as well as their derivatives (Rababah, Hettiarachchy, & Horax, 2004; Rajaei, Barzegar, Mobarez, Sahari, & Esfahani, 2010).

3.4. α-Amylase and α-glucosidase activities

α-Amylase and *α*-glucosidase are two of the enzymes (pancreatic and intestinal, respectively) responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides, which are the compounds suitable for absorption. The inhibition of these enzymes may be a good strategy to regulate the metabolic alterations related to type 2 diabetes and hyperglycaemia (Nowicka et al., 2016). Therefore, in this study, the inhibition of these enzymes was measured in pistachios and results are presented in Table 3 as IC_{50} (mg of dried nut mL^{-1}).

All the samples studied showed very low inhibition values for both enzymes, which indicated the great inhibitory power of pistachio nuts. Although the differences among samples for both enzymes studied were very small (less than 5 units), significant statistical differences were found. In the case of α-amylase inhibition, "Kerman" and "Mateur" samples showed the lowest IC_{50} value of all the studied cultivars (5.49 and 5.56 mg of dried nut mL^{-1}, respectively), followed by "Larnaka" and "Aegina" (6.56 and 6.71 mg of dried nut mL^{-1}, respectively). The rest of the samples presented higher values, with "Napolitana" and "Sirora" samples having the highest values, with an average value of 10.5 mg of dried nut mL^{-1}. Similar results were observed in case of the α-glucosidase enzyme, in which, again, the samples that presented a lower IC_{50} value were "Kerman", "Mateur", "Aegina" and "Larnaka" samples (values lower than 0.05 mg of dried nut mL^{-1}).

To the best of our knowledge, the α–amylase and α-glucosidase inhibitory activity of pistachios nuts, comparing the cultivar effect, has not been previously evaluated and reported elsewhere. Therefore, the data presented in this study can be considered as the first report in the literature stating this point.

3.5. Characterization of volatile compounds

It is important to highlight that fresh pistachio samples were analyzed in this study, and that most of the compounds characteristic of pistachio flavor are developed during roasting of the nuts. Thirteen volatiles compounds were isolated in fresh pistachio fruits by HS-SPME GC-MS (Table 4): 2 alcohols (1-methoxy-2-propanol and 1-hexanol), 2 aldehydes (hexanal and nonanal), 6 terpenes (α-thujene, α-pinene, β-pinene, *trans*- β-ocimene, limonene and terpinolene), 1 pyrrole (1-methylpyrrole) and 2 additional compounds (acetic acid and benzyl acetate). *α*-Pinene was the most abundant compound (109-333 mg kg^{-1} of dried nut) in all samples, followed by 1-methylpyrrole (13–48 mg kg^{-1} of dried nut). The content of these two main compounds represented ~94% of the total volatile molecules; thus, it can be concluded that they are key compounds in the characteristic odor of this nut. The sensory descriptors of these compounds are related to woody and

herbaceous notes (Table 4). Aegina and Napolitana samples had the highest concentration of total volatile compounds (375 and 370 mg kg^{-1} of dried nut, respectively) due to, mainly, they were the two samples that had the highest concentration of *α*-pinene (333 and 321 mg kg^{-1} of dried nut, respectively). The small differences in the contents of the minor compounds could be behind the small differences in the odor, aroma and flavor of these pistachio samples. For example, the small but statistically significant differences observed in hexanal, β-pinene, and limonene can be used to explain the more complex sensory profile of some of the samples.

These results agreed with those obtained by other researchers such as Hojjati, Calín-Sánchez, Razavi, and Carbonell-Barrachina (2013) and Penci et al. (2013), who also obtained that *α*-pinene was one of the predominant compounds in the aromatic composition of pistachio and its essential oil.

3.6. Sensory analysis

Out of the 25 sensory attributes studied (Table 5), significant differences were found in 23 of them (Table 6). Only in the attributes bitter and astringent, there were no significant differences, probably due to the fact that the intensity of these attributes was very low in this specific nut.

Considering the visual attributes studied in the pistachio samples (Table 6), the "Kerman" and "Mateur" samples were characterized by the whiteness of their shell and the uniformity in the fruit size, which make them very attractive for consumers. On the other side, samples of the cultivars "Kastel" and "Sirora" were characterized by the dark color of their shell. As for the color of the nut skin, "Avdat" and "Larnaka" samples had the most intense purple color, while "Kerman" and "Kastel" were the samples that presented the lowest intensity of this color and instead had an intense green color (Table 6).

Regarding flavor, "Avdat" stood out mainly because of its sweetness, with an intensity of 6.0 *versus* an average intensity of 3.2 for the rest of the samples (Table 6). This high sweetness found in this sample, predominated over the rest of the flavor attributes studied (nut flavor, pistachio ID, and aftertaste) and perhaps masked their sensory perception and intensity, leading to lower values as compared to the other cultivars. "Kerman" sample showed the highest intensity of key attributes of nut flavor (6.8), pistachio-ID (7.9), and aftertaste (6.6), (Table 6), attributes that are very important when assessing the sensory quality of this nut, because they are buying- and satisfaction-drivers, according to our own experience with nut consumers. In a similar study carried out by Tsantili et al. (2010), the "Kerman" cultivar was preferred by consumers among other varieties, because it had the best

sensory profile. These results are directly related to those obtained in the analysis of the volatile composition of the samples. In this, "Kerman" sample had a slightly but statistically significant higher concentration of limonene, which is a key compound in the aroma of this fruit because it gives aromatic citrus notes.

Regarding texture, all studied samples presented a characteristic dry nut texture; that is to say, the samples were hard, crispy, had a certain intensity of cohesiveness and adhesiveness, at the same time that they lacked almost completely juiciness or oiliness in the mouth. "Larnaka" and "Aegina" samples were the most representative of this type of texture; that is, they had the highest intensity of hardness and crispiness (these are the texture attributes most appreciated by consumers (Carbonell-Barrachina et al., 2015).

4. CONCLUSIONS

This was the first study stablishing the relationship between pistachio cultivar with its functional properties – mainly related to phenolics - and sensory attributes. After comparing the results obtained, it has been demonstrated that pistachios of the cultivar "Larnaka" had the highest contents of polyphenols and polymeric procyanidins, while samples of the cultivars "Aegina", "Avdat" and "Sirora" had the lowest ones. Besides, samples of the cultivars "Kerman" and "Larnaka" had the highest values of antioxidant activity as well as high inhibitory effect of *α*-amylase and *α*-glucosidase; on the other hand, "Sirora" had the opposite behavior. Regarding flavor, "Kerman" was the cultivar with the highest flavor intensity, which might be linked, among other factors, with slightly higher limonene concentration. "Kerman" and "Mateur" samples stood out for their skin whiteness, while "Avdat" and "Larnaka" had the most intense skin color (purple). Finally, "Aegina" and "Larnaka" had the highest intensities of key texture attributes, including hardness and crispiness. Consequently, it is not easy to make a final recommendation on which cultivar is the best one, and this recommendation must be done according to specific objectives; for instance, if pistachio nuts with high functionality or healthy properties are required, "Larnaka" and "Kerman" are the best options. Whereas, "Kerman" is most suitable if flavor characteristics are demanded.

ACKNOWLEDGEMENTS

The authors are grateful to the projects AGL2013-45922-C2-1-R and AGL2013-45922-C2-2-R (Ministerio de Economía y Competitividad, Spain). Luis Noguera-Artiaga was funded by an FPU grant from the Spanish government (FPU014/01874). Authors want to thank Dr. M.O. Cortez-Rocha for his help.

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Figure1. External appearance of samples of different cultivars of pistachios

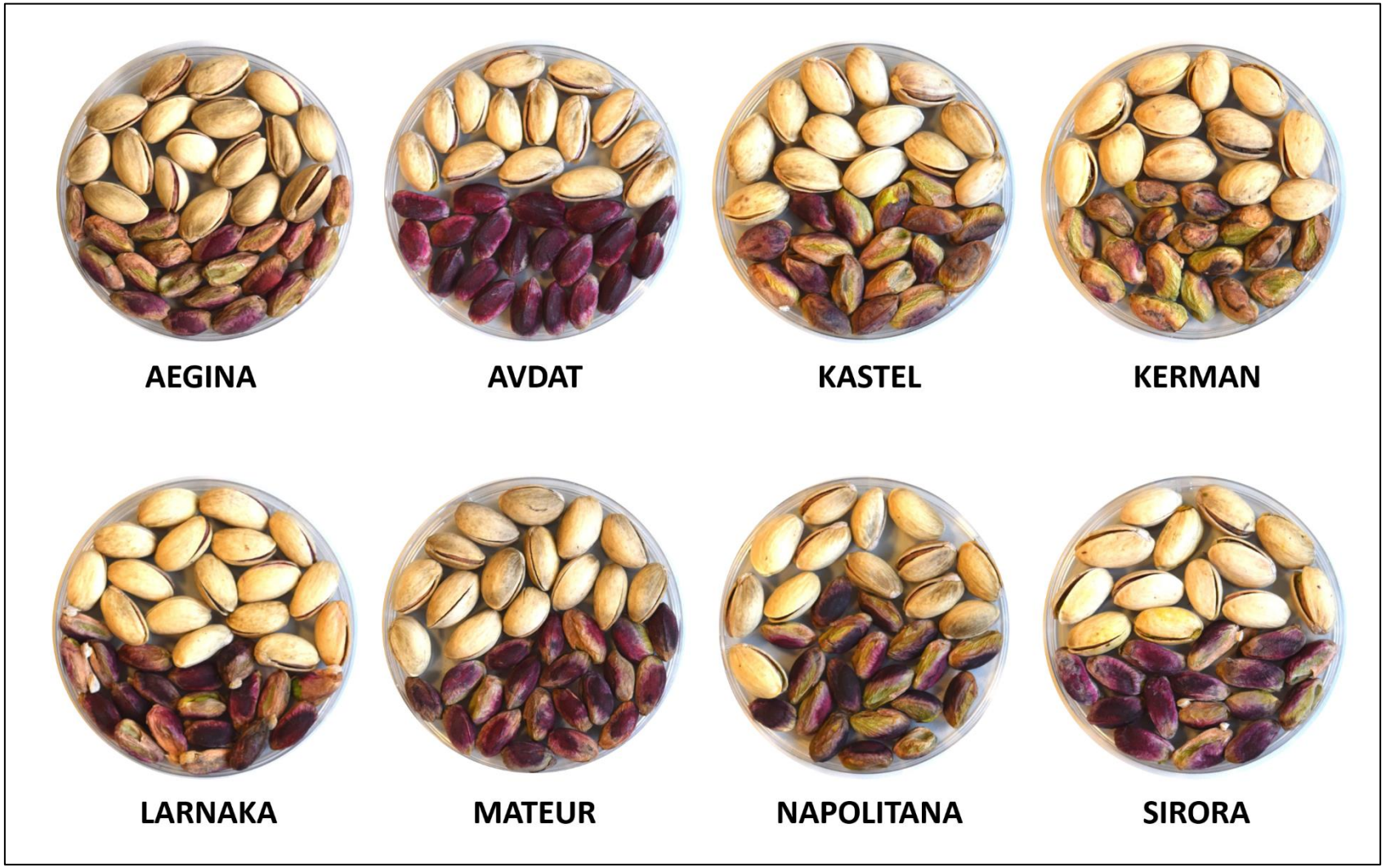

Table 1. Identification of phenolic compounds found in pistachios nuts using LC-MS-QTof/PDA.

Compounds	Retention time (min)	λ_{max} (nm)	MS $[M-H]^-$ (*m/z*)	MS/MS (*m/z*)
Gallic acid	1.76	269	168.97	
Protocatechuic acid	2.74	258, 293	152.97	
Cyanidin-3-*O*-galactoside	3.68	287, 517	449.07	287.11
Procyanidin dimer	3.92	274	577.06	289.02
Kaempferol-3-*O*-hexoside	4.14	272, 350	447.02	284.98
(-)-Epicatechin	4.28	270	289.01	245.04
(-)-Epicatechin-*O*-hexoside	4.31	283	451.06	289.02/323.07
Myricetin-*O*-xyloside	5.34	254,305,366	449.03	317.05/281.09
Myricetin-*O*-galloyl-deoxyhexoside	5.86	266, 356	615.18	463.12/317.05
Myricetin-*O*-hexoside	5.87	269, 356	479.13	317.05
Quercetin-3-*O*-rutinoside	6.61	350	609.06	301.02
Quercetin-3-*O*-galactoside	6.78	346	463.02	301,02
Eriodictyol-3-*O*-glucoside	6.88	283	449.03	287.12
Kaempferol-3-*O*-hexoside	6.97	352	447.02	285.05
Quercetin-triglucoside	10.04	354	625.20	301.01

Table 2. Quantification of phenolic compounds (mg 100 g^{-1} dw) in different pistachio cultivars.

Compounds	ANOVA[†]	Aegina	Avdat	Kastel	Kerman	Larnaka	Mateur	Napolitana	Sirora
		(mg 100 g^{-1} dw)							
Gallic acid	**	3.48 ab[‡]	3.90 ab	2.65 b	2.96 ab	6.64 a	4.07 ab	2.91 ab	2.37 b
Protocatechuic acid	***	3.03 bc	5.12 ab	2.36 bc	3.45 bc	6.86 a	2.12 c	2.35 bc	1.76 c
Keampferol-3-*O*-hexoside	***	3.47 a	0.64 c	1.10 bc	1.17 bc	2.29 ab	1.14 bc	0.72 c	1.05 bc
Myricetin-*O*-xyloside	***	6.08 c	27.12 a	2.24 c	3.32 c	22.26 ab	5.78 c	9.40 bc	5.47 c
Myricetin-*O*-galloyl-deoxyhexoside	***	1.91 c	8.13 a	1.13 c	1.96 c	6.56 ab	2.42 c	2.51 c	3.05 bc
Myricetin-*O*-hexoside	**	6.67 b	9.99 a	2.72 c	2.13 c	11.46 a	1.86 c	4.45 bc	1.98 c
Quercetin-3-*O*-rutinoside	NS	1.04	9.52	4.80	4.29	9.02	5.56	8.77	3.67
Quercetin-3-*O*-galactoside	**	1.79 abc	2.95 ab	1.04 c	1.45 bc	3.46 a	2.37 abc	1.88 abc	1.95 abc
Eriodictyol-3-*O*-glucoside	*	0.72 b	7.70 a	0.34 b	0.40 b	2.96 ab	0.85 ab	1.03 ab	0.91 ab
Kaempferol-3-*O*-hexoside	NS	5.68	11.37	3.16	4.46	8.35	4.93	9.03	6.94
Quercetin-triglucoside	NS	6.26	5.46	3.91	5.69	10.63	5.32	4.42	5.73
Cyanidin-3-*O*-galactoside	*	27.01 b	130.59 a	12.29 c	16.35 bc	55.92 b	27.36 b	46.95 b	27.41 b
Polymeric Procyanidins[¥]	***	505.72 e	348.13 e	4600.97 b	1348.94 d	5919.35 a	1585.23 d	3824.68 c	437.80 e
Total	***	572.85 e	570.65 e	4638.70 b	1396.57 d	6065.77 a	1649.01 d	3919.24 c	500.11 e

[†]NS: not significant at $p< 0.05$; *, **, and ***, significant at $p< 0.05$, 0.01, and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same raw, were not significantly different ($p< 0.05$), Tukey's least significant difference test. [¥]Polymeric procyanidins included sum of Procyanidin dimer, (-)-Epicatechin, (-)-epicatechin-O-hexoside and other polymeric flavan-3-ols.

Table 3. Antioxidant activity and determination of α-amylase and α -glucosidase inhibition IC_{50} (mg dried nut mL^{-1}) of pistachio cultivars.

Pistachio cultivar	ABTS^{+} (mM Trolox 100 g^{-1} dm)	FRAP	α-Amylase [IC50 (mg mL^{-1})]	α-Glucosidase
ANOVA test[τ]	***	***	***	***
Aegina	4.397 d[‡]	2.506 b	6.71 d	<0.05 d
Avdat	4.562 cd	3.191 ab	7.41 c	2.49 c
Kastel	1.521 e	1.090 cd	8.41 b	5.94 a
Kerman	6.210 a	3.191 ab	5.39 e	<0.05 d
Larnaka	5.881 ab	3.617 a	6.56 d	<0.05 d
Mateur	0.402 f	1.515 c	5.56 e	<0.05 d
Napolitana	5.276 bc	3.306 ab	10.21 a	3.67 b
Sirora	0.456 f	0.257 d	10.99 a	3.69 b

[τ] ***: significant at $p<0.001$. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and same factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

Table 4. Concentration of volatile compounds (mg kg^{-1}), retention time, RT (min), Kovat's index (KI), and sensory descriptors of pistachio cultivars.

Compound	Sensory descriptor	RT (min)	KI	ANOVA†	Aegina	Avdat	Kastel	Kerman	Larnaka	Mateur	Napolitana	Sirora
					Concentration (mg kg^{-1})							
Acetic acid	Vinegar	3.529	682	*	3.39 bc‡	2.02 c	2.07 c	1.97 c	4.54 b	1.57 c	6.21 a	6.11 a
1-Methoxy-2-propanol	*nf*	4.282	720	NS	1.85	2.11	1.06	1.62	1.83	1.37	1.50	0.52
1-Methylpyrrole	Woody, smoky, herbaceous	5.516	782	**	15.25 c	18.03 c	27.41 b	48.67 a	45.06 a	22.65 bc	31.57 b	13.67 c
Hexanal	Fatty, green	6.877	835	**	9.19 a	0.29 c	0.78 c	3.08 b	0.35 c	0.49 c	1.71 c	0.86 c
1-Hexanol	Green, herbaceous, woody	9.231	912	*	1.07 a	1.26 a	0.23 b	1.23 a	0.31 b	0.12 b	0.12 b	0.43 b
α-Thujene	Woody, green, herbal	10.262	938	NS	0.56	0.68	0.08	0.25	0.53	0.38	0.14	0.24
α-Pinene	Woody	10.651	948	***	333 a	185 c	109 d	281 b	255 b	183 c	321 a	201 c
β-Pinene	Woody	12.636	999	**	6.57 b	5.45 b	0.96 e	3.63 d	8.98 a	4.72 c	3.35 d	3.12 d
trans-*β*-Ocimene	Green, tropical, woody	13.794	1025	NS	0.39	0.87	0.33	0.34	0.85	0.18	0.21	0.45
Limonene	Lemon, orange, citrus	14.740	1046	*	0.98 d	1.03 d	1.65 c	3.79 a	2.42 b	1.64 c	2.80 b	2.45 b
Terpinolene	Plastic	17.381	1103	*	0.29 b	2.08 a	0.31 b	0.07 b	0.25 b	0.30 b	0.30 b	0.90 b
Nonanal	Fruity, citric, rose	19.381	1144	NS	0.68	0.99	0.58	1.20	0.63	0.09	0.17	0.14
Benzyl acetate	Fruity, floral	22.696	1212	*	1.79 a	1.63 a	0.56 c	1.67 a	1.19 b	0.87 b	1.17 b	0.46 c
Total				***	375 a	222 c	146 d	349 b	322 b	218 c	370 a	230 c

Rt = Retention time; nf = not found; †NS: not significant at $p< 0.05$; *, **, and ***, significant at $p< 0.05$, 0.01, and 0.001, respectively.

‡ Values (mean of 3 replications) followed by the same letter, within the same raw, were not significantly different ($p< 0.05$), Tukey's least significant difference test.

Table 5. Appearance, flavor and texture attributes, definitions, and references used in the study.

Attributes	Definition	References and intensities
Visual		
Shell color	Visual evaluation of the color intensity of the shell.	Pantone 451C = 8.0
Stains	Percentage of the shell with different color of the general (not own fruit color).	50 % = 10
Thickness	Thickness (equatorial diameter) of the sample.	18 mm = 8.0, 10 mm = 2.0
Length	Length of the sample.	22 mm = 9.0, 15 mm = 2.0
Uniformity	Degree of similarity of size between samples.	80 % = 8.0; 50 % = 1.0
Shape	Three-dimensional fruit size ratio (thickness/length).	1 = 10; 0.5 = 2.5
Texture	That has wrinkles or roughness on its surface and is rough to the touch.	Almond shell = 9.0; Peanut kernel = 1.0
Peel	Ease or difficulty when removing the shell of the fruit.	Oh! Nuts pistachio (roasted) = 9.0; Oh! Nuts pistachio (raw) = 5.0
Skin color	Visual evaluation of the color intensity of the skin.	Pantone 261C = 10; Pantone 460C = 2.0
Kernel color	Visual evaluation of the color intensity of the kernel.	Pantone 4645C = 9.0; Pantone 460C = 2.0
Kernel shape	Three-dimensional kernel size ratio (thickness/length).	1.0 = 10; 0.5 = 2.5
Roughness (amount)	Amount of wrinkles on its surface.	Almond shell = 9.0; Peanut kernel = 1.0
Roughness (type)	Depth of skin wrinkles.	Deep = 10; Shallow = 1
Flavor		
Sweet	The taste stimulated by sugars and other sweet substances.	Sucrose solution 5 g/L= 6.5
Bitter	The taste stimulated by substances such as quinine or caffeine.	Caffeine solution 0.5 g/L= 1.5
Astringent	The puckering or shrinking of the mouth caused by substances such as alum or tannins.	Alum solution 1.5 g/L = 1.5
Nut flavor	The nut-like aromatic that is typical of several different nuts such as almonds and hazelnuts.	Borges *Selección Mezcla de Frutos secos* = 8.0
Pistachio ID	Aromatics reminiscent of pistachio.	Oh! Nuts pistachio = 8.0
Aftertaste	Time it remains in the mouth the characteristic flavor of pistachio after swallowing or expectorating the sample.	5 s = 1.0; 20 s = 10
Texture		
Hardness	The force required to bite completely through the sample with molar teeth. Evaluate on first bite down with the molars.	Carrots fresh = 7.5; Cheese American Land O'Lakes = 3.0
Crispiness	The intensity of audible noise at first bite with molars.	Cheerios = 5.0
Juiciness	The sensation of moisture released by the pistachios during the first two bites.	Raw carrot (peeled) = 1.5
Oiliness	The amount of oily coating perceived on the mouth after swallowing or expectorating.	Potato chips Lay's = 8.0
Cohesiveness	The amount the sample deforms rather than cuts during first two bites.	Baby food-bananas = 7.0; Granny Smith apple= 2.0
Adhesiveness	The degree to which product sticks on the surface of teeth.	Mushrooms fresh unpeeled = 2.0; MilkyWay bar= 8.5

Table 6. Descriptive analysis of pistachios obtained by different cultivar

Attributes	ANOVA[†]	Aegina	Avdat	Kastel	Kerman	Larnaka	Mateur	Napolitana	Sirora
Visual									
Shell color	***	6.2 b[‡]	6.0 b	3.5 e	6.7 a	5.0 c	7.1 a	6.0 b	4.0 d
Stains	***	4.7 c	6.7 a	5.0 bc	5.0 bc	4.2 c	2.2 d	5.0 bc	5.7 b
Thickness	***	3.0 d	3.2 d	5.4 b	7.0 a	3.4 d	2.7 d	3.0 d	4.2 c
Length	***	5.0 c	5.4 bc	7.0 a	7.0 a	5.2 bc	5.0 c	6.7 a	5.9 b
Uniformity	***	8.7 cd	9.2 bc	8.5 d	9.5 ab	7.7 e	10.0 a	8.2 de	8.7 cd
Shape	***	3.0 de	3.2 de	6.7 b	9.0 a	2.9 de	3.3 d	2.8 e	4.2 c
Texture	***	2.7 bc	2.2 c	2.2 c	3.2 bc	2.5 c	4.0 ab	5.0 a	4.0 ab
Peel	***	7.5 c	9.4 ab	8.5 bc	9.7 a	10.0 a	8.5 bc	7.9 c	9.7 a
Skin color	***	8.2 d	9.2 b	5.2 e	4.5 f	9.9 a	7.8 d	8.2 cd	8.7 bc
Kernel color	***	3.0 d	6.5 a	2.0 e	2.0 e	3.7 bc	4.0 b	6.1 a	3.2 cd
Kernel shape	***	3.2 de	3.7 cd	6.2 b	8.1 a	3.0 e	3.4 cde	3.0 e	4.0 c
Roughness (amount)	***	3.2 bc	1.7 de	4.9 a	2.2 cd	1.0 e	4.0 ab	4.4 ab	1.9 de
Roughness (type)	***	2.5 b	1.5 c	1.9 bc	2.7 ab	1.2 c	2.7 ab	3.5 a	2.2 bc
Flavor									
Sweet	***	2.7 b	6.0 a	3.5 b	2.5 b	3.5 b	3.3 b	4.2 b	2.7 b
Bitter	NS	1.5	1.0	1.3	1.0	1.2	1.0	1.1	1.2
Astringent	NS	1.1	0.5	0.8	0.7	0.7	0.7	0.9	0.9
Nut flavor	*	6.6 ab	5.8 c	6.1 bc	6.8 a	7.0 a	6.0 bc	6.3 b	6.4 b
Pistachio ID	***	5.8 bc	5.5 c	6.3 bc	7.9 a	5.8 bc	5.5 c	6.8 ab	5.5 c
Aftertaste	***	5.5 abc	5.1 c	6.5 ab	6.6 a	5.5 bc	4.9 c	5.9 abc	5.6 abc
Texture									
Hardness	***	6.3 ab	5.7 b	5.4 b	5.1 b	7.5 a	5.2 b	5.3 b	5.5 b
Crispiness	**	4.7 a	3.5 ab	3.8 ab	3.3 ab	4.5 a	3.6 ab	3.0 b	3.0 b
Juiciness	**	3.1 ab	3.5 a	3.3 a	1.9 b	2.5 ab	3.0 ab	3.1 ab	3.7 a
Oiliness	***	6.0 a	5.5 ab	5.4 ab	2.7 c	5.5 ab	3.6 bc	4.2 abc	5.5 ab
Cohesiveness	**	6.5 a	6.3 a	6.4 a	4.9 c	6.1 ab	5.2 bc	5.7 abc	6.0 ab
Adhesiveness	**	2.9 a	3.0 a	2.4 b	3.0 a	3.0 a	2.2 b	2.8 a	2.5 b

[†]NS: not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. [‡]Values (mean of 3 replications) followed by the same letter, within the same row, were not significantly different ($p < 0.05$), Tukey's least significant difference test.

Capítulo 2

Concepto de hidrosostenibilidad

PUBLICATION 2

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Journal of Food Science, 81(10), S2559-S2565, 2016.

DOI: https://doi.org/10.1111/1750-3841.13501

Índice de calidad

Journal Citation Reports (2016)

Área: Food Science & Technology

Factor de impacto: 1.815 (52/130)

Cuartil: Q2

OPINION OF SPANISH CONSUMERS ON HYDROSUSTAINABLE PISTACHIOS

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ABSTRACT

Fruits and vegetables cultivated under controlled deficit irrigation (CDI) are called hydrosustainable (hydroSOS) products and have its own personality and are environmentally-friendly. Focus groups helped in classifying key farming, sensory, and health concepts associated with CDI-grown pistachios. Besides, focus groups also helped in stating that a logo was needed for these special foods, and that a hydroSOS index is also essential to certify that the products have been controlled by a control board. Conjoint analysis was used to check which attributes could be helpful in promoting CDI-grown pistachios among Spanish consumers in a first step towards the European Union (EU) market. It was clearly proved that the main silo of properties driving the attention of Spanish consumers was that related to health. The most important attributes for pistachios were "product of Spain", "rich in antioxidant", and "crunchy"; this finding was clearly related to the popularity of regional foods, the preoccupation of European consumers for their health, and the joy related to the crunchiness of toasted nuts, respectively. The use of these three concepts, together with the use of the hydroSOS logo, will be essential to promote hydroSOS pistachios among Spanish and EU consumers. Finally, it is important to highlight that in general Spanish consumers were willing to pay an extra amount of 1.0 euros per kg of hydroSOS pistachios. These earnings will be essential to convince Spanish farmers to implement CDI strategies and have a sustainable and environmental-friendly use of the irrigation water.

PRACTICAL APPLICATIONS: The information generated in this study will be essential for farmers as a starting point for promoting their hydroSOS pistachios, and a similar strategy can be implemented for other hydroSOS vegetables and fruits. Nowadays, it is necessary to address consumers' demands to ensure new products' success in the market.

KEYWORDS: conjoint analysis, controlled deficit irrigation, focus group, *Pistachia vera*, water efficiency.

1. INTRODUCTION

The world food production heavily depends on water availability; in fact it depends more on water than on any other type of abiotic stress (Grant 2012). Due to political and environmental reasons, an increase in the availability of water will be quite difficult. Thus, it will be more and more necessary to improve water efficiency; that is, the commercial production *per* unit of applied water (Kijne and others 2003), by implementing new farming technologies (Katerji and others 2008).

A sustainable use of water is the one that does not reduce, on a net basis, the quantity or the quality of this natural resource. Under such conditions and because it is almost impossible to significantly increase water availability, even by using recycled wastewater, the needs must be specially controlled (Fereres and González-Dugo 2009). The strategy for the reduction of needs implies: (i) the management of smaller or deficit quantities of irrigation and/or (ii) the use of drought resistant species. Controlled deficit irrigation (CDI) implies the application of water deficit conditions in non-critical periods of the crop to minimally affect crop production and quality (Chalmers and others 1981). Fruits and vegetables cultivated under CDI, "hydrosustainable or hydroSOStainable" (hydroSOS from now on) products, will have a solid identity. This identity will be based on two facts: (i) water stress will make plants to increase their secondary metabolites, leading to increases in key compounds that can improve the quality and functionality of these products (higher content in bioactive compounds, higher intensity of some sensory attributes, etc.) (Ripoll and others 2014), and (ii) they will be environmentally friendly because the use of a very valuable resource in the world, water, will be optimized in their production.

After the application of CDI in the field, hydroSOS products will be obtained and they must be distinguished from those that have received non-deficit irrigation (Cano-Lamadrid and others 2015; Carbonell-Barrachina and others 2015). In this study pistachio nuts were used as a model of hydroSOS product obtained after CDI, especially because of the high consumer liking for this type of nuts; however, our research team is working with other vegetal products, such as table olives (Cano-Lamadrid and others 2015), and pomegranates (Galindo and others 2014), and similar strategies will be used to differentiate them from their conventional counterparts. Pistachio tree (*Pistacea vera* L.) is considered as one of the most draught and salinity resistant fruit species (Behboudian and others 1986). Around 4,000 ha are dedicated in Spain for cultivation of pistachio (Gijón and others 2009), mainly in the provinces of Ciudad Real and Toledo. This surface makes Spain one of the countries in the EU (basically a pistachio-importing region) with a higher cultivated area, and because of similarities between pistachios and almonds (Spain

is one of the biggest world producers of almonds), pistacho cultivation in Spain has a huge potential.

Water relations and irrigation scheduling have not been studied deeply in pistachio trees. However, the sensitivity of the phenological stages of the crop has been already established; the shell hardening stage is the most resistant one to water stress (Gijón and others 2009), and it is related to changes in the elasticity modulus and the capacity of osmotic adjustment (Gijón and others 2011). On the other hand, the stage of seed growing is the most sensitive to water stress (Goldhamer and Beede 2004). Finally, Memmi and others (2016) has concluded that as much as 43-73% of the irrigation water can be saved by using soft of moderate CDI strategies combined with the use of Ψ_{stem} (midday stem water potential) to manage irrigation scheduling. With the irrigation conditions reported in this study (Memmi and others 2016), no fruit yield reduction was observed.

In a context of change of food habits, the interest of consumers in products with high quality and/or "own personality", including eco-friendly products (hydroSOS pistachios will have their own personality because of their eco-friendly use of irrigation water), has increased in the developed countries. Almli and others (2011) have recently showed that European consumers believe that the effort made in the production of "special" products, including efforts made to control water stress in plants, is compensated by their specific sensory attributes and their healthy characteristics. The consumers' perception of the quality of foods with "own personality" greatly depends on their personal preferences, cultural and religion influences (Iaccarino and others 2006), demographic and psychological characteristics (Ophuis and van Trijp 1995), product authenticity perceived by the consumer (Kuznesof and others 1997), and quality of expectations. This last factor can be also affected by the geographical origin (Stefani and others 2006), price (Lange and others 1999), nutritional information (Kähkönen and Tuorila, 1998), traditional making technology (Rason and others 2007), and, probably, by cultural conditions, such as how much water has been used for its production (e.g. type of irrigation).

After consideration of the above information, the main aim of this study was to determine the main aspects of pistachios grown under CDI driving consumers purchase intention in order to be able to emphasize these concepts when later promoting the consumption of the later labelled hydroSOS pistachios. Results of the present study will help farmers in successfully promoting any type of hydroSOS vegetal products, including pistachios.

2. MATERIALS AND METHODS

2.1. Focus group

Four 60-min focus groups were conducted, with at least 10 consumers *per* group, who typically consumed pistachios or almonds at least 1 to 2 times per week, to establish the key attributes that consumers expect in pistachios cultivated under controlled deficit irrigation (CDI) strategies. A total of 45 persons were interviewed, covering a wide range of ages (18 to 65), with 63% of the participants being female. Participants were recruited from the SensoFood Solutions (http://sensofoodsolutions.umh.es) consumer database, and were screened to ensure they: (i) ate nuts (almonds and/or pistachios), (ii) were not associated with agriculture or food science related industries, and (iii) had lived over 10 years in Spain or the European Union. The focus groups were conducted in the facilities of the Polytechnic Superior School of Orihuela (EPSO), Orihuela, Alicante (Spain).

A trained focus group moderator managed the focus group discussions, which included topics related to (i.) consumer habits (e.g.: if you only had to purchase food items for yourself, compared to purchasing for the whole family, would you have different concerns?), (ii) health (e.g.: what are the special characteristics you look for when choosing a healthy food item? ("light", "high fiber content", etc.), (iii) agricultural practices, including irrigation systems (e.g.: what do you think about information relative to the nuts production system, for example: products labeled as "sustainable"? Should they have information about the use or non-use of pesticides on crops, irrigation system used, etc.? Is this information important to you when choosing a product?), and (iv) sensory properties of different nut products including pistachios (e.g.: what is the main sensory property you think of when thinking of nuts and nut products?). Additional issues raised by the consumers during the group sessions were also discussed; however, the concept of "hydroSOS" products was not discussed during these sessions because it was still under creation. At the very beginning of each group session, definitions of nuts and irrigation water strategies were provided to the consumers; these explanations were provided to ensure that all consumers had initially the same knowledge on nuts and the use of water in agriculture.

The focus groups were recorded and all responses and terms from the participants were first collected, and later organized (**Table 1**) by a group of six highly experienced professors of the Universidad Miguel Hernández de Elche, who belong to different knowledge areas (Vegetal Production, Agriculture, Environment, Food Science and Technology, and Sensory Analysis). The grouping of concepts was later used in the conjoint analysis.

2.2. Conjoint analysis

Conjoint analysis is used frequently in industry and research for measuring consumer trade-offs among behaviors associated with purchasing or adopting products and services (Krieger and others 2003; Mahanna and others 2009; Mahanna and Lee 2010). This statistical tool works by combining simple variables into more complex concepts according to an experimental design (Krieger and others 2003). Jaeger and others (2011) provide additional background on the technique.

In the present study, the concepts obtained from the focus groups, after proper grouping, were used for the conjoint analysis (**Table 1**). The components of the silos [(terms/elements of the 3 mentioned categories: *farming* (3 elements), *sensory* (3 elements), and *health* (5 elements)] were systematically varied to create different combinations with all 3 components belonging to different silos (total of 45 combinations). A total of 45 paper cards were prepared; each card presented one of the aforementioned created combinations. Two examples of a "card concept" are shown in **Figure 1**. The question asked to the consumers was: *How likely would you be interested in purchasing pistachios with the characteristics shown in the card?* Consumers participating in the conjoint analysis experiment were evaluating pistachios in general, and they received no information about hydroSOS products or CDI strategies. A total of 90 consumers were recruited from the SensoFood Solutions database to evaluate the cards.

A completely randomized design was used with a 5% confidence level (α) and fixing the statistical power (1-β) of the test at 80%; under these conditions a sample size, according to the expected differences among the factors of n=15 cards for each individual. Thus, each consumer received an e-mail and rated 15 cards; it was considered that a consumer could rate 15 cards without losing the interest and keeping maximal concentration. A total of 1350 ratings *per* product were obtained (30 ratings *per* concepts' combination *per* product). The cards were rated on an "interest scale" from 0 to 10, with 0 being "no interest at all" and 10 being "I would definitely buy it", with increments of 0.5 units. The card presentation was randomized causing all consumers to have different combinations of 15 cards to evaluate. A demographic questionnaire was also administrated at the consumers at the end of the survey, and included the following questions: (*i*) sex (male, female), (*ii*) range of age (18-24, 25-35, 36-45, 46-55, >56), (*iii*) are you in charge of buying foods? (yes, no), (*iv*) are you a frequent consumer of organic foods? (yes, no), (*v*) how often do you eat nuts (almonds and/pistachios)? (daily, several times a week, once a week, 2-3 times per month, 1 or less per month), and (*vi*) where do you eat nuts (almonds

and/or pistachios)? (in pubs together with a drink, at home as appetizer, as ingredient in foods, in meetings/parties with friends, others).

Regression with dummy variables was conducted using PROC REG of SAS® (version 9.2; SAS Inst., Cary, N.C., USA) for consumer data to predict the interest score of each concept. Dummy variables regression was used because the elements evaluated were discrete rather than continuous. Additionally, conjoint analysis was also conducted using PROC TRANSREG of SAS® to obtain the part-worth utility of each item and the relative importance of attributes.

The obtained matrix was used to perform linear regression analysis illustrating the drivers for acceptance as positive coefficients in a regression equation and the potentially "dangerous" elements that drive rejection as negative coefficients in a regression equation.

2.3. Sensory Evaluation with Consumer panel and Willingness to Pay

Commercial pistachios were purchased from Mercadona supermarkets (Ourense and Alicante, Spain) and were labeled as "hydroSOStainable" or "conventional" products; this is, the exact same pistachios were presented using two different labels. Pistachios samples were evaluated by 100 consumers, from two different locations: (i) Ourense (Galicia Community, Spain), and (ii) Alicante (Valencia Community, Spain). The main reason to choose these two locations was that the regions of Galicia and Valencia are characterized by having high and low water availability, respectively; thus, the initial hypothesis was that consumers in Valencia should be more aware of the importance of water for the society, especially for agriculture, than those from Galicia.

The whole group of consumers (n=200) consisted of 54 % women and 46% men, aged between 20 and 63 years old. The main requirement for their recruitment was that they consume nuts (almonds and/or pistachios) at least once a week; and an additional recruitment criterion was that they should be responsible for buying foods at their households. Consumers tested the two pistachio samples, coded with 3-digit numbers, in one session; the samples order for each consumer was randomized. Approximately 5-6 pistachios were served, at room temperature together with the appropriate questionnaire, one at a time, and waiting 5 minutes between samples. Unsalted crackers and water were provided to consumers for palate cleansing between samples.

To check the isolated power of the logo hydroSOStainable, consumers evaluated their products without any additional explanation at the beginning of the

test. Future studies will be performed checking the power of the logo together with the information related to hydroSOS farming.

In each questionnaire, consumers were asked, using 9-point hedonic scales [from 0 to 9, with 5 (the central point of the scale) being "neither like nor dislike it"], about their "global" satisfaction degree, and also their satisfaction degree for saltiness and crunchiness. Finally, a question about their willingness to purchase was also included at the end of the questionnaire. The options for the price of a 125 g bag of pistachios were: 1.25, 1.50, 1.75, 2.00, 2.25, and 2.50 euros. The normal prices of the Mercadona pistachios ranges from 1.75 to 2.00 euros *per* 125 g bag, depending on the season. Thus, there were two prices above and two below the normal market price of this product.

Results from this study are provided as the mean ± standard error. First, data were subjected to two-way [factor 1 = treatment (conventional or hydroSOS); factor 2 = location (Galicia or Valencia)] analysis of variance (ANOVA), and later data was also subjected to LSD multiple-range test to compare the means. Differences were considered statistically significant at $p<0.05$. All statistical analyses were performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD).

3. RESULTS AND DISCUSSION

3.1. Focus groups

After the organization of the terms collected during the group sessions, 4 categories or "silos" were found: (i) terms related to *farming* (production of vegetable or fruits), (ii) terms related to *health* (bioactive components of foods and their effects on human health), (iii) terms related to *sensory* attributes of foods, and (iv) terms related to *price*. The willingness to pay for hydroSOS pistachios has been evaluated in this study as a separate experiment; thus, the category on *price* was eliminated from further testing, and 3 silos for the conjoint analysis were included: (i) *farming*, (ii) *health*, and (iii) *sensory*. Authors want to have all the information about the opinion of Spanish consumers on these categories (farming, health, and sensory) to be able to finally get the best possible price for the hydroSOS products; but this last stage will be done with the help of the logo and marketing campaigns based on the results from these 3 categories. Thus, each of these three silos contained only discrete, but not excluding, terms (**Table 1**), rather than quasi-continuous variables with levels. In this way, selecting one concept from one silo did not mean the others were excluded, but that they were less important for the consumer in driving its purchase intention. A similar strategy (discrete terms) was also the choice taken by

Vázquez-Araújo and others (2012), when developing new products based on sorghum. However, other researchers prefer to use a combination of discrete and continuous variables (Kimura and others 2011).

Initially all terms mentioned by the consumers were listed within a particular category or silo; after that, redundant terms were eliminated, and similar or complementary concepts were combined into a single term, representing the general ideas shown by consumers. The final list is shown in **Table 1** and includes 3, 5, and 3 terms within the categories of farming, health, and sensory, respectively.

One of the first things mentioned by the consumers during the group sessions was the need to develop strategies to distinguish CDI products among the ones already in the market; according to some of the consumers "there is a need *to stand out in the crow*". In this sense, consumers mentioned that the best way to provide key information regarding the products is to include it in the food package; also a high percentage of people indicated that television, newspapers, and radio (in decreasing order) would be good options to provide information to consumers.

The first strategy developed to distinguish CDI products, in this particular case pistachios, was to design a logo for this category of "new" vegetal products (**Figure 2**), later labelled as hydroSOS. This activity was conducted in parallel to the conjoint analysis experiment, that will be later discussed; this is the main reason why the logo was not used in that analysis. However, in the next studies with other fruits or vegetables, the hydroSOS will be definitely included in the conjoint analysis studies to get a full image of the opinion of the consumers on the logo. The logo was designed in two different languages English and Spanish, because the marketing campaigns will start in Spain, but will be later extended to other countries of the European Union in English, if successful in Spain. As it can be seen, there is a central message (SOS), in the middle of the logo, on the scarcity of water and the need to efficiently use this crucial resource.

Besides, it would be interesting to develop a "hydroSOS index" to certify that pistachios with the hydroSOS logo have been evaluated regarding their hydrosustainability or the way farmers have limited irrigation water. This index should consider factors such as the following ones: (i) knowledge of the crop, including water management at the less sensitive growin stages, (ii) strengh and duration of the water stress, (iii) practical control of the stress, by considering for example how many times the farmer has measured the water potential, etc. Our research team is currently working on establishing this hydroSOS index, but still needs further research before it is finally ready. Besides and according to the

available research on the effects of CDI on pistachio (Memmi and others 2016), it can be stated that high quality (nutritional, functional, and sensory) hydroSOS pistachios can be produced after cultivation with 45% (range from 43-70%) less water than in control trees (commercial irrigation strategies).

When considering pistachio *farming* issues, a high percentage of the participants gave not too much importance to concepts such as organic farming, sustainability or eco-friendly strategies; this was especially true for old participants. However, they were willing to pay a slightly higher price for pistachios grown locally or nationally. On the other hand, young participants showed higher interest in the concepts related to the environment and also at the same time for national products or even more for local foods. It is possible that terms such as "sustainable" are not fully understood by the "oldest" consumers; for instance, Farley and Smith (2002) indicated the concept of sustainability is a widespread goal that nearly everyone supports, but almost no one means the same thing when they use the term. In this way, the importance of this concept is underestimated especially by old consumers. However, other researchers (Stolz and Bautista 2015) reported that older consumers showed a significant interest in the environmental impact of their purchases.

The fact that this list has a higher number of terms within the *health* category (5), as compared to 3 in the other two categories, showed the high attention that nowadays consumers pay to the healthy aspects of food (Zhu and others 2016; Hemmerling and others 2015). There is a high number of scientific references supporting the beneficial effects of nuts (Hernandez-Alonso and others 2014; Dreher 2012), including pistachios, and their main components: (i) high energy (Kirbaslar and others 2012), (ii) cholesterol-free (Dreher 2012), (iii) high contents of minerals (Schlörmann and others 2015; Kirbaslar and others 2012), (iv) high content of compounds with antioxidant capacity (Schlörmann and others 2015; Liu and others 2014), and (v) rich polyunsaturated fatty acid profile (Schlörmann and others 2015; Kirbaslar and others 2012).

3.2. Conjoint analysis

Initially and considering only the mean values of the 45 combinations, the best 6 results were obtained for the combinations showed in **Table 2**; all had means above 7.5. The soundness of this ranking is supported by the fact that this same order is shown for both the median and the mean.

Later and after the application of the conjoint analysis, it can be stated that the silo "health" promoting properties of pistachios in general was the most important

category for 43.8% of the consumers participating in this study. From this it can be stated that health promoting properties will be essential to advertise hydroSOS pistachios. This marketing strategy is also supported by previous studies by Carbonell-Barrachina and others (2015) who found that hydroSOS pistachios (stem water potential < -1.3 MPa) showed a fatty acid profile with higher contents of linoleic acid (polyunsaturated and essential fatty acid) than controls, and also showed higher intensities of key sensory attributes, and a greater satisfaction degree among international consumers (Spain, Poland, France, and Slovak Republic). Further research on the effects of RDI on the functionality (full polyphenols profile, antioxidant activity, and α-amylase inhibition activity) of hydroSOS pistachios is on-going and results will be available in a near future.

The other two factors, "farming" and "sensory" properties, showed similar relative importance, 28.2 and 28.0%, respectively (**Figure 3**). This result is particularly logical because nowadays consumers are very worried about their health and pay big attention to the health information in labeling (Wasowicz and others 2015), marketing and advertisement (Lin 2015). Pohjanheimo and Snadell (2009) reported that age and education were related to food choice motives. Adult consumers (28 to 41 years old) had a university degree and considered food choice motives such as health (e.g. "keeps me healthy"), ethical concern (e.g. "comes from countries I approve politically", and natural content (e.g. "contains no additives") to be highly important. It is important to mention that 67% of the participants in the present study had an age between 25 and 45 years old, and thus are within the age range of adult consumers.

The part-worth utility values for the studied concepts are shown in **Figure 4**. The choice "product of Spain" was the most appreciated farming characteristic. This result from the survey agreed quite well with the comments obtained during the focus groups work, in which a high percentage of participants mentioned that "local foods" were appealing for them. Feldman and Hamm (2015) have concluded that local food is not perceived as expensive, on the contrary to organic food, and this leads to more positive opinions towards this type of products. Besides, consumers stated that they were willing to pay extra-money for products clearly labeled as local because they want to support local farmers and economy. Probably this is the result of previous policies encouraging consumers to buy more locally produced foods; this was the case of the UK government (Chambers and others 2007). Another factor that could be related to the relative importance of consuming local foods is the so called spatial localization (food miles) that has emerged as a dominat issue in environmentally benign alternative food systems (Cleveland and others 2015). On the other hand,

factors such as "eco-friendly" or "sustainable" were less appreciated by Spanish consumers. In the authors' opinion, concepts such as "eco-friendly" or "sustainable" are still not "fully" understood by the general population and will not key in driving their food choice. These two terms (eco-friendly and sustainable) were included in the conjoint analysis to evaluate this hypothesis and check whether they were able to drive consumers' attention and whether the later authors' hypothesis was right or not. Withing the "health" silo, the fact that pistachios are a source of energy ("with the energy of nuts") did not drive consumers' willigness to buy pistachios, perhaps because of the worries of consumers regarding fatty products with high levels of energy (**Figure 4B**). On the opposite side, the concepts "rich in antioxidants" and "healthy fatty acids profile" were positively valued by Spanish consumers. These two concepts are supported by recent clinical studies proving the positive effects of nuts on the human health, especially cardiovascular disease (Ros 2014, 2015). Thus, these two health claims may be useful when promoting pistachios in Spain and in the European Union.

Finally, the most important factor related to the sensory properties of pistachios was "crunchy", and also the concept "fun-to-eat" positively affected the opinion of consumers on pistachios. However, the concept of "toasted and salty combination" was not too attractive for the consumers, perhaps because of including the word "salty" which seems not too popular at this time. In this way, even there is a big controversy regarding the risks associated to salt consumption (Graudal and others 2014; Frisoli and others 2012), it is generally admited that consumers want to reduce their salt intake and actions are being taken (Zandstra and others 2016). Consequently, it is important than during processing of pistachios it is ensured that they are as crunchy as possible, and the fun associated to its consumption seems also important, and if possible low salt is also highly recommended. However, the social desirability of having toasted and "salty" pistachios in a pub together with a drink is a completely different matter; under that specific situation, the senses will be under control, and the salty side of the pistachios will enhance the pistachios flavor and will help in enjoying them.

3.3. Willingness to pay

HydroSOS products has a competitive disadvantage compared to traditional products because they should cover (i) the reductions in yield by limiting irrigation water, and (ii) the increase of costs due to changes in the irrigation systems, as mentioned by Ingenbleek (2015).

The willingness to pay for pistachios was affected by both the irrigation information (conventional *versus* CDI) and the geographical location (Galicia or Valencia) (**Table 3**). Spanish consumers were willing to pay a significantly ($p<0.01$) higher price for hydroSOS pistachios as compared to conventional samples. In fact, they were willing to pay 1.72 € *per* bag of 0.125 kg, which is equivalent to a final price of 13.76 € kg^{-1} for hydroSOS nuts, as compared to 12.72 € kg^{-1} for the conventional ones; this values led to a difference of 1.04 € kg^{-1}. The consumers from Galicia were willing to pay a significantly ($p<0.05$) higher price for the pistachios than those from the Valencia region, exactly 13.60 as compared to 12.88 € kg^{-1}. The initial hypothesis of this study was that Valencia consumers should be willing to pay higher prices because water is scarcer there than in Galicia; however, the real situation was exactly the opposite one. This experimental finding could be due to the fact that all consumers in Spain are aware of the importance of the water, and the drastic reduction in the availability of irrigation water. However, further studies, using audiction methods, will be performed to make a more complete evaluation of the willingness to pay of Spanish and European consumers for hydroSOS products, including pistachios. Besides, differences in the consumers' willigness to pay before and after receiving information about the main characteristics of hydroSOS products will also be assayed in future studies.

ACKNOWLEDGEMENTS

The authors are grateful to the projects AGL2013-45922-C2-1-R y AGL2013-45922-C2-2-R (Ministerio de Economía y Competitividad, Spain), and want to thank: (i) Marina Cano-Lamadrid for her help in the designing of the product logos, and (ii) Dr. Marisa Speziale for her help in the focus groups. Luis Noguera-Artiaga was funded by a FPU grant from the Spanish government (FPU014/01874).

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Table 1. Silos and concepts chosen for the conjoint analysis of hydro-sustainable pistachios.

Farming	Sensory	Health
Product of Spain	Crunchy texture	With the energy of nuts
Sustainable	Toasted and salty combination	Cholesterol-free
Eco-friendly	Fun-to-eat	Rich in minerals
		Rich in antioxidants
		Healthy fatty acids profile

Table 2. Six concept combinations getting the highest values in the conjoint analysis.

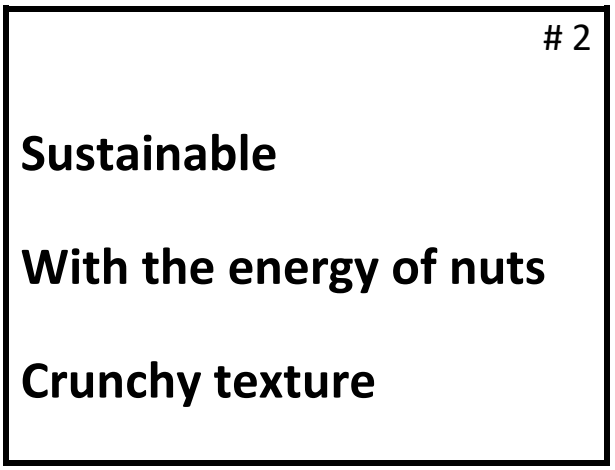
Farming	Sensory	Health	Mean	SD	Min	Median	Max
PRODUCT OF SPAIN	**CRUNCHY TEXTURE**	**RICH IN ANTIOXIDANTS**	7.9	1.6	5.0	8.0	10.0
Sustainable	**CRUNCHY TEXTURE**	With the energy of nuts	7.7	1.2	6.0	8.0	10.0
PRODUCT OF SPAIN	**CRUNCHY TEXTURE**	Healthy acids profile	7.7	1.4	4.0	7.8	10.0
PRODUCT OF SPAIN	Toasted and salt combination	Healthy acids profile	7.7	1.4	4.0	7.5	10.0
Sustainable	Toasted and salt combination	**RICH IN ANTIOXIDANTS**	7.6	1.3	5.5	7.5	10.0
Eco-friendly	**CRUNCHY TEXTURE**	**RICH IN ANTIOXIDANTS**	7.5	1.6	3.5	7.5	10.0

Table 3. Overall liking and satisfaction degree on saltiness and crunchiness and willingness to pay for pistachio samples as affected by irrigation treatment and geographical location of the study.

Factor	Overall	Saltiness	Crunchiness	Willingness to Pay
ANOVA Test[†]				
Treatment	NS	NS	NS	**
Location	**	*	NS	*
Treat. × Locat.	NS	NS	NS	NS
LSD Multiple Range Test[‡]				
TREATMENT				
Conventional	6.6	6.0	6.4	1.59 b
HydroSOS	6.9	6.0	6.5	1.72 a
LOCATION				
Galicia	6.5 b	5.7 b	6.6	1.70 a
Valencia	7.0 a	6.3 a	6.4	1.61 b

[†] *NS = not significant at p< 0.05; *, **, and ***, significant at p< 0.05, 0.01, and 0.001, respectively.* [‡] *Values followed by the same letter, within the same column and factor, were not significantly different (p<0.05), according to LSD multiple range test.*

Figure 1. Example of 2 of the cards showed to the respondents of the conjoint analysis survey.



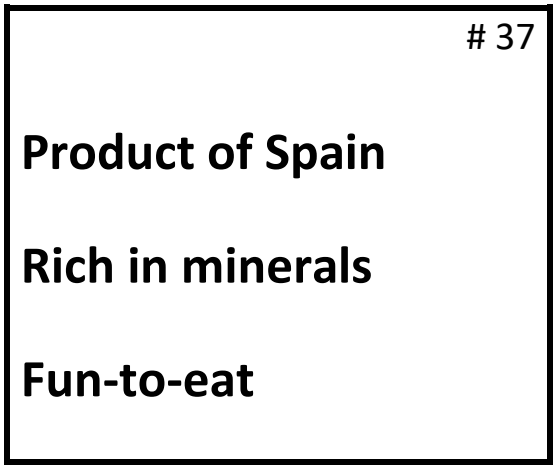


Figure 2. First logo designed for the differentiation of hydro-sustainable products in Spanish and English-speaking countries.

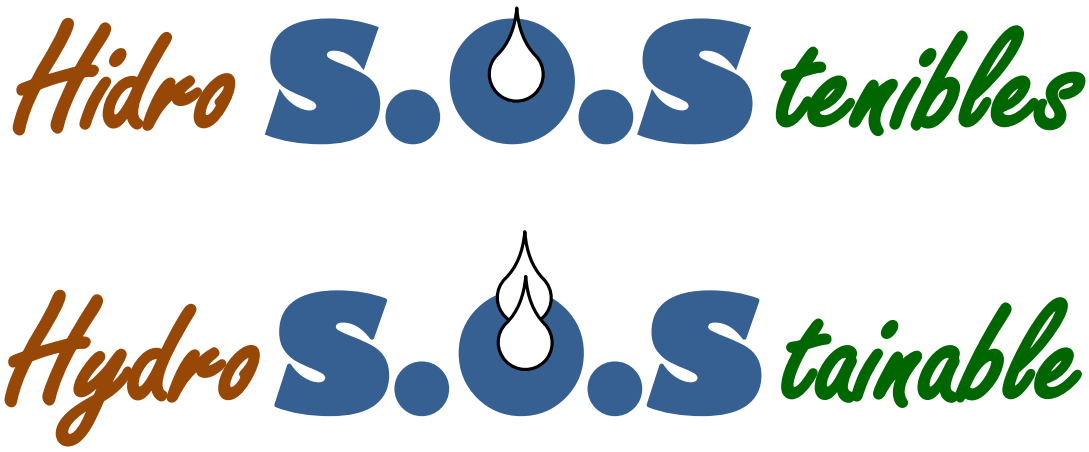


Figure 3. Average importance of each of the silos or categories involved in the conjoint analysis of hydro-sustainable pistachios.

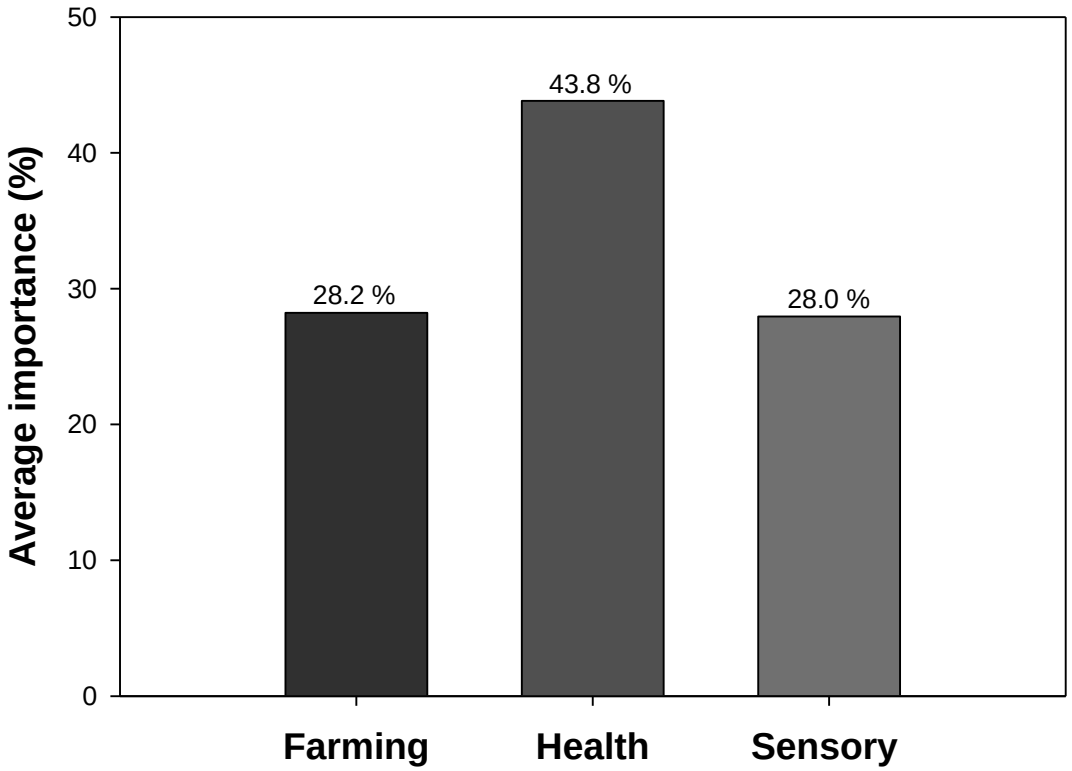
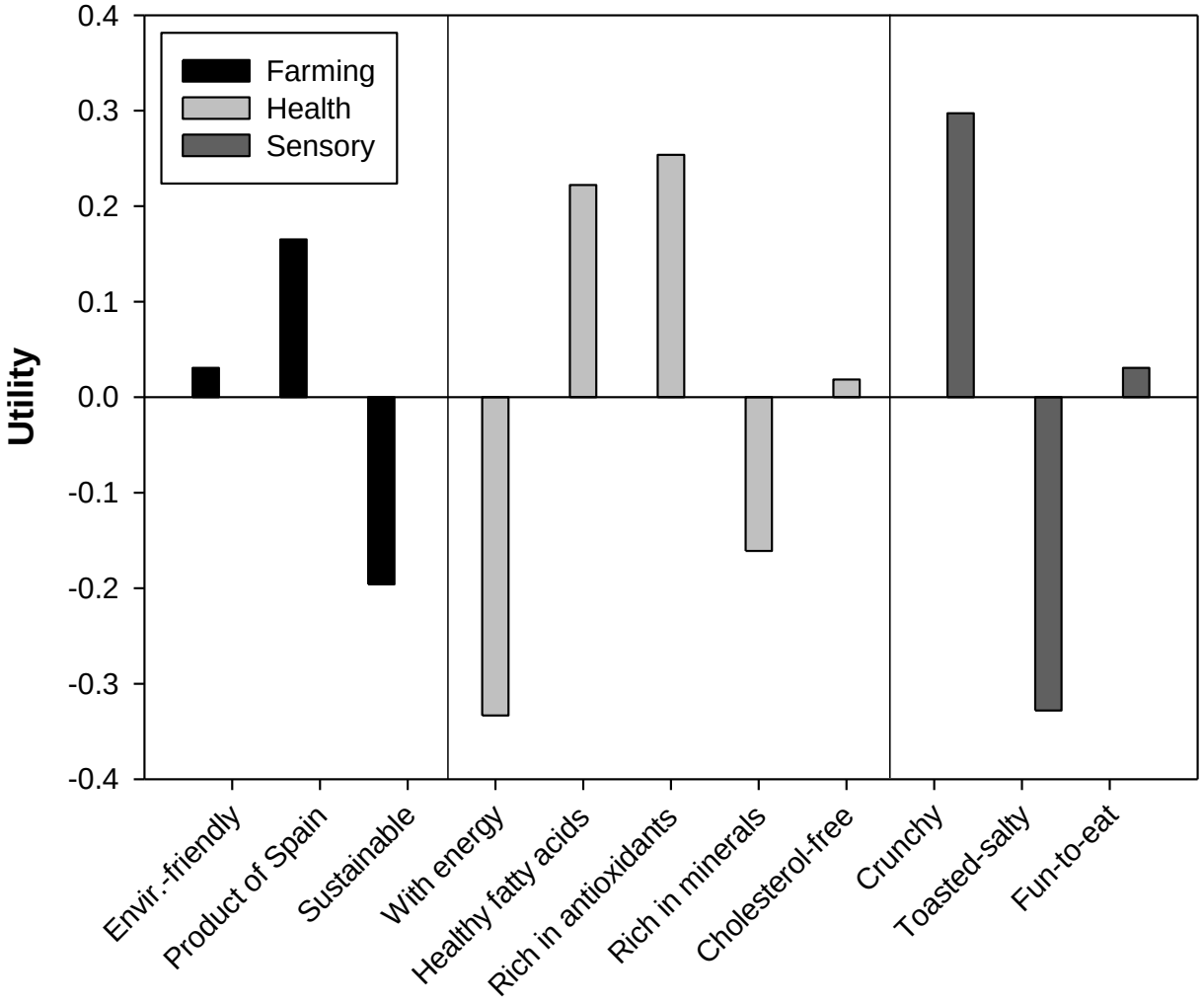


Figure 4. Part-worth utility values for concepts related to hydro-sustainable pistachios.



Capítulo 3

Calidad físico química y sensorial de pistachos hidrosostenibles

PUBLICATION 3

INFLUENCE OF REGULATED DEFICIT IRRIGATION AND ROOTSTOCK ON THE FUNCTIONAL, NUTRITIONAL AND SENSORY QUALITY OF PISTACHIO NUTS

Luis Noguera-Artiaga, Paola Sánchez-Bravo, Francisca Hernández, Armando Burgos-Hernández, David Pérez-López, Ángel A. Carbonell-Barrachina

Scientia Horticulturae, 2019

(Aceptado para su publicación)

Índice de calidad

Journal Citation Reports (2018)

Área: Horticulture

Factor de impacto: 1.961 (5/36)

Cuartil: Q1

Date: 10/29/2019
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Influence of regulated deficit irrigation and rootstock on the functional, nutritional and sensory quality of pistachio nuts
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INFLUENCE OF REGULATED DEFICIT IRRIGATION AND ROOTSTOCK ON THE FUNCTIONAL, NUTRITIONAL AND SENSORY QUALITY OF PISTACHIO NUTS

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Declaration of interest: none

ABSTRACT

The aim of this study was to determine the influence of rootstock and regulated deficit irrigation (RDI) on pistachio nuts, by studying their size, weight, color, fatty acids (gas chromatography with mass spectrometer detector), minerals (atomic absorption), total polyphenol content (TPC), antioxidant activity (ABTS, FRAP, and DPPH), and sensory properties (trained panel). Three rootstocks (*P. atlantica*, *P. integerrima*, and *P. terebinthus*) and three irrigation treatments (T0: fully irrigation, 100 % ET_c; T1: during phenological phase II the stem water potential, SWP, was maintained around −1.5 MPa; and T2: during phase II, SWP < −2.0 MPa) were used. Pistachios obtained from *P. terebinthus* had the highest size, weight and oleic acid content (main fatty acid), while *P. integerrima* nuts had the best sensory profile. The use of moderate RDI (T1) led to pistachio nuts with higher weight, smaller size, similar fatty acid profile, higher TPC (1284 and 1192 g GAE kg^{-1} dry weigh, respectively), and similar (no statistically differences) antioxidant activity (AA) and sensory profile than control samples. Thus, moderate RDI produces nuts with a good functional quality (high values of TPC and AA), without affecting their sensory quality, but being environmentally friendly and having reduced economic cost due to a lower use of irrigation water.

KEYWORDS: Antioxidant activity; fatty acid methyl esters; hydroSOS; *Pistacia vera*; total polyphenol content.

1. INTRODUCTION

The pistachio tree (*Pistacia vera*) belongs to the Anacardiaceae family, and it is considered the only commercially edible nut among 11 species of the *Pistacia* genus (Couceiro et al., 2013). Thus, *P. vera* is the species with the greatest commercial interest and as a consequence it is widely cultivated in Mediterranean countries (Galindo et al., 2018).

Traditionally, pistachio trees are considered resistant to drought and salinity (Goldhamer, 1995), although the effect of the water stress on the quality and especially on the functionality of the nuts has not been fully characterized. The current water scarcity is forcing farmers to focus on crops that can withstand water deficit. Thus, pistachio is a good alternative for arid or semi-arid farming areas. This crop could generate important advantages for farmers, decreasing water consumption and, therefore, increasing economic benefit and income, as well as being beneficial for the biodiversity of the ecosystem (Pérez-López et al., 2018). Following this recommendation, at the end of the 1980 decade, regulated deficit irrigation (RDI) was used in pistachio trees grafted on *P. atlantica* in California (Goldhamer et al., 1987). RDI is an irrigation strategy through which the tree is subjected to water deficit at specific phenological stages, considered less sensitive, and without significantly affecting the yield or the economic benefits (Behboudian and Mills, 1997; Gijón et al., 2011). Pistachio nuts are characterized by 3 phenological stages: stage I, the nut starts its growth; stage II, the shell hardens (stage less sensitive to irrigation deficits); and stage III, the kernel grows (Fereres et al., 2003; Gijón et al., 2011).

Fruit and vegetables cultivated under RDI are marketed under the brand "hydroSOStainable or hydroSOS" products. This brand is characterized by being environment-friendly (optimized use of irrigation water) and by a theoretical increase of secondary metabolites, which will improve the quality and functionality of the RDI products (Noguera-Artiaga et al., 2016).

On the other hand, pistachio is a species with low rooting capacity; it is not possible to propagate this species by cutting and planting because not enough roots are produced. Thus, it is necessary to use rootstocks for its vegetative propagation (Moriana et al., 2018). Studies on pistachio rootstocks are not numerous and, in general, they are focused on comparing the productive response (yield) and/or diseases resistance. Selection of the rootstock is one of the most important decisions for the development of a pistachio orchard, and the appropriate rootstock could be different for different farming areas. *P. atlantica*, *P. integerrima,* and hybrids between *P. atlantica* x *P. integerrima* are the main rootstocks in USA; *P. francs* are used in Turkey; while *P. mutica*, *P. khinjuk* and *P. francs* are the most used in Iran (Acar et al., 2017).

Considering all the above-discussed information, the aim of this work was to study the influence of regulated deficit irrigation (RDI) and the use of different rootstocks on the quality (morphological, physico-chemical, functional and sensory properties) of pistachio nuts.

2. MATERIALS AND METHODS

2.1. Plant material, growing conditions and experimental design

Pistachio nuts from trees (*P. vera*), cultivar "*Kerman*" were collected during 2015 from the experimental orchard "La Entresierra" located at Ciudad Real, Spain (3°56' W, 39° N; altitude 640 m above sea level). The climate of this area is Mediterranean, with an average annual rainfall of 397 mm. The soil is a shallow clay-loam (Petrocalcic Palexeralfs) with a discontinuous petrocalcic horizon located at 0.5 m with a pH about 8.1, low electrical conductivity (0.2 dS m^{-1}), 1.05 % organic matter, 0.12 % N, $17x10^{-4}$ mol kg^{-1} K and a high cation exchange capacity (0.186 mol kg^{-1}).

A completely randomized factorial design was used for the study. There were 18 plots in the field, with 2-replicates and 2-factors: (i) irrigation treatment, and (ii)

rootstock. Each experimental plot consisted of 2 trees which were used for the measurements, surrounded by 10 trees under the same conditions (rootstock and irrigation).

Three irrigation treatments were evaluated:

(i) T0 (control), in which trees were irrigated to ensure non-limiting water conditions in the soil (100 % ET_C of the previous week, estimated according to daily reference evapotranspiration calculated using the Penman-Monteith equation, a crop factor based on the time of the year, and taking into consideration the canopy size (Allen et al., 1998; Fereres and Goldhamer, 1990; Goldhamer, 1995);

(ii) T1, during phase II irrigation was suppressed until pistachio trees reached a stem water potential (SWP) below −1.5 MPa; then, irrigation was managed to keep SWP below this threshold but near of it; and

(iii) T2 had the same irrigation protocol as T1 but with a SWP threshold of −2.0 MPa.

Treatments T1 and T2 were irrigated with a threshold of -1.1 MPa, considerate as without water stress during phenological phases I and III.

Water relations were characterized according to Memmi et al. (2016): irrigation was performed daily using a drip irrigation system with 12 self-compensating emitters (each delivering 4 L h^{-1}) per tree; the irrigation water used had an electrical conductivity of 2.6–2.9 dS cm^{-1}. Irrigation started when the measured SWP values were lower than the targeted threshold values. The first irrigation event was always 1 mm. Then, each increase or decrease of irrigation water supply was scheduled according to the percentage of difference derived from the relationship between measured SWP (0.25 mm day^{-1} for every 10 % deviation). The soil moisture was measured using a portable capacitance probe (Diviner 2000 Sentek Pty. Ltd., Stepney South, Australia) using the default calibration supplied by the manufacturer, according to procedure described by Memmi et al. (2016).

Besides, pistachio trees were grafted over 3 different rootstocks: *P. atlantica*, *P. integerrima* and *P. terebinthus.*

Pistachio nuts were collected from the field, peeled and dried in a convection oven with hot air at 60 °C until the targeted moisture content of 5 % (~3 d) was reached. Nuts were immediately packed and ~2 kg were posted to Universidad Miguel Hernández de Elche, Orihuela (Alicante, Spain) for analyses. Finally, samples were vacuum packed and kept under refrigeration (4-5 °C) until analysis.

2.2. Morphological analysis

Twenty-five pistachio nuts from each treatment were randomly selected and the whole nut, shell and edible part were weighed (model AG204 scale; Mettler Toledo, Barcelona, Spain) with a precision of 0.1 mg. Also, length, width and height of the edible part from each pistachio taken per treatment were measured using a digital caliper (model 500-197-20 150 mm; Mitutoyo Corp., Aurora, IL, USA).

Color was measured using a colorimeter (model CR-300, Minolta, Osaka, Japan) with an illuminant D65 and an observer of 10 °. Twenty-five pistachios were ground for 10 s (Taurus Aromatic Ver II; Taurus Group, Barcelona, Spain) and placed in Petri dishes (100x15 mm). Color data were provided as CIE$L^*a^*b^*$ coordinates and the following coordinates were evaluated: L^* (lightness); a^* (green-red coordinate) and b^* (blue-yellow coordinate). Lightness taking values within the range 0–100, the coordinate a^* takes positive values for reddish colors and negative values for greenish ones, whereas b^* takes positive values for yellowish colors and negative values for bluish ones. Besides, C^* (Chroma, $C^* = [(a^{*2}) + (b^{*2})]^{1/2}$ was studied. Chroma 0 is at the center of a color sphere and increases according to the distance from the center. Analyses were run in triplicate (using 25 nuts for each measurement).

2.3. Fatty acids

The determination of fatty acid methyl esters (FAME) were carry out following the method described by Carbonell-Barrachina et al. (2015), with some modifications. The organic layer of the extracts was identified and quantified using a gas chromatograph, Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detect or GCMS QP-5050A, equipped with a polar column, Suprawax 280, 100 % polyethylene glycol (Teknokroma S. Co. Ltd., Barcelona, Spain; 30 m x 0.25 m x 0.25 μm film thickness). Detector and injector temperatures were 260 °C and 230 °C, respectively. Helium was used as a carrier gas at a flow rate of 1.1 mL min^{-1} (split ratio 1:10). The oven program was as follow: initial temperature 80 °C (hold 2 min) and rate up to 160 °C (8 °C min^{-1}); then, rate up to 220 °C (rate of 4 °C min^{-1}; hold 13 min); and, to finally, rate up the oven temperature up to 260 °C (rate of 10 °C min^{-1}; hold 6 min). Identification of peaks was made by comparison with FAME standards from Sigma-Aldrich. Analysis of FAME was run in triplicate (n=3).

2.4. Mineral content

To determine the mineral content in the samples, 0.5 g of ground pistachios were digested for 2 hours in a multi-place digestion block (Digest 20, Selecta), at a temperature below 130 °C, using 5 mL of 65 % (w/v) HNO_3 as described Carbonell-Barrachina et al. (2002). Then, dilutions of 1:10 and 1:50 were prepared and stored in refrigeration (4 °C) until analysis. The quantification of minerals was carried using an atomic absorption–emission spectrometer (Solaar 969; Unicam Ltd, Cambridge, UK) using atomic absorption for Ca, Mg, Cu, Fe, Mn and Zn, whereas atomic emission was used for K, and Na. Calibration curves were used for the mineral quantification and they showed good linearity ($R^2 \geq 0.997$). Five replicates were run for the mineral analysis.

2.5. Antioxidant activity

It is necessary to use several of methods to better compare the results obtained on the analysis of the antioxidant activity, and their choice is based on the matrix of the sample and on the chemical nature of the compounds to be evaluated (Prior et al., 2005). In this work, $ABTS^+$, FRAP, and DPPH* methods were used to describe the antioxidant activity of the samples.

The $ABTS^+$ [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation was carried out as described by Re et al. (1999), ferric reducing antioxidant power (FRAP) as described by Benzie and Strain (1996), and, the radical scavenging activity using the DPPH• radical (2,2-diphenyl-1-picrylhydrazyl) as described by Brand-Williams et al. (1995).

For the analysis, a methanol extract was prepared for each treatment and rootstock. Ten milliliters of MeOH/water (80:20, v/v) plus 1% HCl were added to around 0.5 g of crushed pistachio. Then, the mixture was sonicated at 20 °C for 15 min and left at 4 °C for 24 h. After that, the extract was sonicated again for 15 min and centrifuged at 10,000 x g during 10 min.

Ten-µL of the supernatant was mixed with 990 µL of $ABTS^+$ or FRAP. After 10 min of reaction, the absorbance was measured at 734 nm for ABTS+ and 593 nm for FRAP method. In the DPPH method, 10 µL of the supernatant was mixed with 40 µL of MeOH and added to 950 µL of $DPPH^•$ solution. The mixture was shaken and placed under dark conditions for 15 min. All the results were expressed as mmol Trolox kg^{-1} dry weight, dw.

2.6. Total polyphenol content

For the total polyphenol content (TPC) determination, a methanol extract was prepared for each sample as previously described for antioxidant activity. TPC was quantified using the Folin-Ciocalteu colorimetric method described by Gao et al. (2000), with some modifications. In a sample of pistachio extracts (0.1 mL) were added 0.2 mL of Folin-Ciocalteu reagent, 2 mL of distilled water and incubated for 3

min at room temperature. Then one-mL of 20 % sodium carbonate was added and incubated again for one hour. The TPC was determined by measurement at 765 nm using an UV-visible spectrophotometer (Helios Gamma model, UVG 1002E). Quantification was carried out according to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), g kg^{-1} (dw).

2.7. Sensory analysis

For the sensory evaluation, was used a trained panel formed by nine panelists (aged 30-65 years; five women and four men) from the research group Food Quality and Safety (Universidad Miguel Hernández de Elche, Orihuela, Alicante, Spain). Each panelist had more than 500 h of testing experience with nuts.

About 10 pistachio nuts were served, to each panelist, in odor-free disposable 90 mL covered plastic cups, coded using three-digit numbers, and at room temperature. Between samples, to clean the panelists' palate were used unsalted crackers and drinking water. The testing room was at ~22±2 ºC and natural illumination was used. Four 2 h sessions were done for the samples evaluation. The total number of samples under analysis was 9 and all were evaluated in each session.

The panel worked with the lexicon developed by Carbonell-Barrachina et al. (2015) with some added attributes: floral, bitter, astringent and wood. The panel used a numerical scale for quantifying the intensity of the pistachio attributes, where 0 represents none and 10 extremely strong, with 0.5 increments.

2.8. Statistical analysis

Data from the analyzes performed on the pistachios were processed by means of an analysis of variance (ANOVA) and by the Tukey's multiple range test. Percentage data were transformed by Arcosen function before statistical analysis. The software StatGraphicsPlus 5.0 Software (Manugistics, Inc., Rockville, Maryland, United States) was used. The significant difference was defined as $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Morphological analysis

The mean values for the split-open and non-split-open pistachios were 52.6 and 42.6 %, respectively; there were not statistically significant differences among treatments (**Table 1**). On the other hand, the factor "rootstock" significantly affected the percentages of split and non-split open pistachios, with *P. terebinthus* having the highest value of split open nuts (60 %) and the lowest one of non-split open ones (35 %).

In general, RDI nuts had higher weights (whole, edible nut and shell) than control, especially T2 nuts (**Table 1**). For example, both RDI treatments (T1 and T2), shared the highest value for edible nut weight (~0.7 g). However, control samples were the biggest ones (length, height and width), except in their length; control had the highest values of height and width (10.516 and 9.021 g, respectively).

Regarding rootstocks, *P. terebinthus* showed the highest values (weight and size) whereas *P. integerrima* and *P. atlantica* having the lowest values of weight and size, respectively (**Table 1**).

Color of the samples did not present statistical significant differences, in most of the parameters studied, as affected by the irrigation treatments and rootstocks. Although, lightness (*L**) and tone (*Hue*) were affected by the irrigation treatments, with T1 having the lowest *L** value (64.05), which was reflected in a slight increase, but not significant, in tone (79.92). As can be seen in **Table 1**, the differences among color coordinates for the studied samples were lower than 2-units for most of the treatments. These authors (Galindo et al. (2015) and Navarro et al. (2011) have concluded that differences smaller than 2 units were imperceptible for the human eye. Even though, differences in these determinations were statistically significant, they did not represented an appreciable color change in the final product. Therefore, it can be stated that the irrigation treatment applied and the rootstock used did not significantly affected pistachio color. Similar results were previously reported by Carbonell-Barrachina et al. (2015), who showed that neither RDI treatments nor

rootstocks did significantly affect the weight, size, split-nuts and color of pistachio nuts.

3.2. Fatty acids

Composition of fatty acids from pistachios under study are presented in **Table 2**. Of the 9 fatty acids (FAMEs) identified, 4 were saturated (SFAs), 3 monounsaturated (MUFAs) and 2 polyunsaturated (PUFAs). The SFAs were myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and arachidic acid (C20:0); the MUFAs were palmitoleic acid (C16:1), oleic acid (C18:1), and eicosenoic acid (C20:1) and finally linoleic acid (C18:2) and α-linolenic acid (C18:3) were the PUFAs.

In general, the abundance of FAMEs followed the order: *C18:1 > C18:2 > C16:0 ≫ C18:0 > C16:1 > C18:3 > C20:1 > C20:0 > C14:0*. The mean values for these compounds in the two RDI treatments (mean of T1 and T2) were 52.97 %, 31.12 %, 12.39 %, 1.33 %, 1.06 %, 0.53 %, 0.34 %, 0.13 % and 0.08 %, respectively; RDI did not significantly affect the FAMEs content.

P. terebinthus had the highest percentage (53.88 %) of the main compound, oleic acid (C18:1), while *P. atlantica* had the lowest one (52.17 %). The second most abundant fatty acid was linoleic acid (C18:2), and *P. atlantica* and *P. integerrima* had the highest contents (31.64 and 31.49 %, respectively), without significant different between them. In case specific of *P. integerrima, the* content of linoleic acid increased proportionately to the water restriction (**Table 2**). If FAMEs are grouped according to their unsaturation degree, it can be observed that *P. atlantica* had the lowest concentration of the MUFAs, 53.6 %, and the combination of RDI T2 and *P. terebinthus* had the highest concentration.

This findings agreed with those reported by Acar et al. (2017), who reported that oleic, linoleic and palmitic acids were the 3 main fatty acids in pistachios. Okay and Sevin (2011) observed that the content of oleic acid increased and the content of linoleic acid decreased in well-irrigated trees. Carbonell-Barrachina et al. (2015) showed that a moderate irrigation treatment produced a significant increase in the

linoleic acid content. In another type of nuts, particularly almonds, Zhu et al. (2015) reported that moderate deficit irrigation did not affect to the lipid content.

3.3. Mineral content

Application of RDI on pistachios influenced their mineral composition (**Table 3**). Pistachios from T2 treatment presented higher contents of Na, Ca and Cu than those of the control nuts, but simultaneously they had lower contents of K and Mn. In case specific of Cu, an increase of water restriction led to an increase of this mineral, independently of the rootstock used. The decrease in irrigation water could cause, among other actions, an accumulation of available Ca and Na contents in the soil-plant system by reducing losses by leaching, leading to a reduced absorption and accumulation of K and Mn. This could be justified by the antagonistic effect that Ca and Na can cause on the rest of minerals; for instance, the antagonism trend between Na and K is one of the most studied in plant metabolism (Carbonell-Barrachina et al., 1997). Regarding rootstock, *P. Integerrima* showed the lowest contents of Mg and of all studied micro-nutrients (Cu, Mn, Fe, and Zn). As has been shown, pistachios are a good source of minerals, especially K with ~9 g kg^{-1}, which contributes to normal functioning of the nervous system, to normal muscle function, and to the maintenance of normal blood pressure (Commision Regulation (EU) No 432/2012). The effect of RDI on pistachio had incidence on the content of K, in case of *P. integerrima* and *P. atlantica*, reducing their content when irrigation water was restricted. In case of P. terebinthus the content of K increased with moderate RDI (T1).

3.4. Antioxidant activity (AA) and total polyphenol content (TPC)

In general, RDI pistachios (T1 and T2) showed higher TPC than that of the control (except in case of *P. integerrima* in which T2 had the lowest content), being those from T1 treatment the ones with the highest content (1284 g GAE kg^{-1} dw). Additionally, RDI had a significant effect on the antioxidant activity measured by both

DPPH and FRAP methods (**Table 4**). The moderate RDI treatment (T1) showed no differences regarding these two AA methods with respect to the control, while under the severe irrigation treatment (T2) the antioxidant activity decreased, especially that quantified by the DPPH method. This effect can be observed for every interaction between irrigation treatment and rootstock. Otherwise, the AA measured by ABTS did not showed significant differences among the irrigation treatments. On the other hand, the rootstock did not affect neither the TPC nor the AA of pistachios.

This result indicates that under high water stress, an important stomatal regulation occurs and the CO_2 produced is used to maintain the primary metabolism. However, under a mild or soft water stress, the CO_2 is redistributed to the formation of secondary metabolites although growth is slightly limited. This behavior has been observed in fruits with a high content of bioactive compounds on which RDI has been apply (Behboudian et al., 2011). In addition, situations of water stress causing an accumulation of antioxidant substances, as a physiological response for the removal or control of the free radicals, have been widely reported (Grant, 2012). Similar results were reported in olives by Gucci et al. (2019), who found that phenolic concentration in RDI treatment was higher than in other water regimes. On the other hand, Cano-Lamadrid et al. (2017) obtained that application of RDI treatments on table olives did not influence neither the AA nor the TPC.

3.5. Sensory analysis

The irrigation treatments significantly affected 5 out of the 15 the sensory attributes under analysis: color, size, pistachio-ID, aftertaste and crunchiness (**Table 5**). With respect to the attributes related with pistachios appearance (color and size), T1 treatment showed no differences with respect to the control, while the T2 had lower color intensity and small size (2.8 and 5.1 *versus* 3.5 and 7.7, respectively). Sample of control irrigation treatment (T0) had high intensity of color in case of rootstock P. integerrima and P. atlantica, while sample T1 was the most intense in case of P. terebinthus. However, RDI in general did not affect the sensory quality of

the pistachio; it only led to slight differences (although statistically significant) in pistachio-ID, aftertaste and crunchiness. The T2 treatment had the lowest intensity of pistachio flavor and aftertaste (6.2 and 6.8, respectively), while it had the highest intensity of crunchiness (T0= 7.0, T1= 6.7, and T2= 7.8). The highest crunchiness intensity may be linked to the fibrousness of the nut, due to low water accumulation in these nuts (T2, the most stressed ones). Similar results were obtained by Galindo et al. (2015), who showed that the texture attributes, including crunchiness, in jujube were increased by severe RDI conditions.

On the other hand, the factor "rootstock" had more effects on the pistachio sensory profile than the factor "irrigation treatment". *P. atlantica* nuts had the highest size as compared to *P. terebinthus* and *P. integerrima*. Among the flavor attributes, nutty (global), pistachio-ID, salty, sweet and aftertaste were significantly affected by the rootstock. *P. terebinthus* showed the lowest score for the attributes nutty, pistachio-ID, salty and aftertaste (6.7, 6.6, 0.4 and 6.9, respectively) but the highest sweetness (3.2). In contrast, *P. integerrima* presented the highest values for nutty, pistachio-ID, salty and aftertaste attributes (7.7, 7.6, 0.8 and 7.6, respectively) and the lowest values for sweet (2.2). *P. atlantica* presented a sensory profile in between those of *P. integerrima* and *P. terebinthus*. Regarding texture, *P. integerrima* and *P. terebinthus* presented high values for hardness and crunchiness (*P. terebinthus* 7.6 and 7.8, respectively; *P. integerrima* 8.1 and 7.8, respectively). Regarding friability, *P. integerrima* and *P. atlantica* showed the highest values (6.6 and 5.7, respectively) and *P. integerrima* had the lowest values for adhesiveness (5.7), which was positive because it is considered as a negative attribute by pistachio consumers.

According to the information related to the interaction between the two studied factors (irrigation and rootstock), crunchiness and hardness was reduced with moderate RDI in case of P. integerrima and P. terebinthus, while for P. atlantica the RDI treatments (T1 and T2) led to obtain pistachios with high intensity these two attributes.

As described by Carbonell-Barrachina et al. (2015), the purchase choice of international pistachio consumers based on the sensory attributes is due to pistachio flavor, saltiness, crunchiness and toasted flavor. Although, Noguera-Artiaga et al. (2016) exposed that international consumers preferred intense crunchy but low salty nuts.

4. CONCLUSIONS

The results obtained in this study demonstrated that moderate deficit irrigation (T1) has increased the TPC without showing statistically significant effects on AA, FAMEs profile, weight, number of open pistachios, color, appearance and texture (crunchiness) of pistachios. The application of a severe deficit irrigation (T2) led to decreases on size, AA, and intensity of pistachio-ID flavor and aftertaste, although it had similar values of many other parameters, such as number of open pistachios, texture, flavor, color, TPC and FAMEs profile and even an increased the weight of the samples. On the other hand, the results established that the choice of the rootstock on pistachio cultivation had no significant influence on color, TPC and AA. *P. integerrima* and *P. terebinthus* resulted in an overall increase of MUFAs, size, and texture, despite showing the worst appearance. Furthermore, *P. integerrima* had the highest pistachio-ID flavor and *P. terebinthus* showed the highest weight and number of open pistachios. These results indicated that the application of moderate deficit irrigation (T1) led to an increase product functionality and quality, without jeopardizing nut yield, weight or size, consequently generating savings of water during cultivation, and resulting in less environmental and economic costs. On the other hand, *P. terebinthus* presented appropriate FAME profile, size, texture and weight; thus, it would be also a good choice for the farmers. As a final recommendation, results obtained here proved that the combination moderate deficit irrigation (T1) and *P. terebinthus* rootstock is the best one according to the nuts composition, functionality and sensory quality.

ACKNOWLEDGEMENTS

Authors want to thank the kindness and selfless help that Dr. M.O. Cortez-Rocha provided to this study.

FUNDING

The authors are grateful to the projects AGL2013-45922-C2-1-R and AGL2013-45922-C2-2-R (Ministerio de Economía y Competitividad, Spain). Luis Noguera-Artiaga was funded by an FPU grant from the Spanish government (FPU014/01874).

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Table 1. Weight, size, color and nature of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Weight (g)			Size (mm)			Color Coordinates					Number *per* 100 units		
	Whole	Edible nut	Shell	Length	Height	Width	*L**	*a**	*b**	*C**	*Hue*	Split open	Non-split open	Others[§]
ANOVA Test[†]														
Treatment	***	***	***	**	***	***	**	NS	NS	NS	*	NS	NS	NS
Rootstock	***	***	***	***	***	***	NS	NS	NS	NS	NS	***	***	NS
Treat. x Rootstock	**	**	**	***	***	***	NS	NS	NS	NS	NS	*	*	NS
Tukey's Multiple Range Test[‡]														
Treatment														
T0	1.350 c	0.704 b	0.646 b	16.635 ab	10.516 a	9.021 a	66.23 a	-6.45	33.39	34.01	79.07 ab	55	39	6
T1	1.382 b	0.728 a	0.654 b	16.476 b	10.335 b	8.798 b	64.05 b	-5.90	33.04	33.58	79.92 a	51	45	4
T2	1.408 a	0.727 a	0.681 a	16.755 a	10.110 c	8.823 b	65.44 a	-6.63	33.34	34.00	78.77 b	52	44	4
Rootstock														
Integerrima	1.343 b	0.705 b	0.638 b	16.417 b	10.351 a	8.937 a	65.00	-6.39	33.89	34.50	79.39	48 b	48 a	4
Atlantica	1.393 a	0.716 b	0.678 a	16.711 a	10.148 b	8.657 b	65.46	-6.37	33.19	33.30	78.98	51 b	46 a	3
Terebinthus	1.404 a	0.738 a	0.666 a	16.739 a	10.463 a	9.047 a	65.25	-6.22	32.69	33.78	79.41	60 a	35 b	5
Treat. x Rootstock														
T0* *P. Integerrima*	1.329 c	0.699 c	0.630 c	16.244 c	10.516 a	9.033 a	66.18	-6.57	34.13	34.76	79.11	49 b	46 a	4
T1* *P. Integerrima*	1.357 bc	0.717 ab	0.639 c	16.420 bc	10.393 b	8.924 ab	63.26	-5.73	33.31	33.83	80.36	46 b	51 a	4
T2* *P. Integerrima*	1.344 bc	0.699 c	0.645 b	16.587 bc	10.144 bc	8.855 b	65.56	-6.86	34.22	34.91	78.70	51 b	46 a	3
T0* *P. Atlantica*	1.357 b	0.692 c	0.665 b	16.997 a	10.284 b	8.856 b	66.19	-6.31	32.65	33.26	79.06	51 b	46 a	3
T1* *P. Atlantica*	1.377 b	0.717 ab	0.660 b	16.234 c	9.973 c	8.323 c	64.23	-6.38	32.64	33.26	78.95	51 b	46 a	4
T2* *P. Atlantica*	1.445 a	0.737 a	0.708 a	16.902 a	10.186 bc	8.793 b	65.98	-6.42	32.77	33.40	78.91	51 b	47 a	3
T0* *P. Terebinthus*	1.363 b	0.721 ab	0.642 c	16.666 bc	10.748 a	9.173 a	66.32	-6.47	33.39	34.01	79.05	62 a	34 b	4
T1* *P. Terebinthus*	1.412 ab	0.749 a	0.664 b	16.774 b	10.638 a	9.147 a	64.66	-5.58	33.16	33.63	80.46	59 a	36 b	5
T2* *P. Terebinthus*	1.436 a	0.744 a	0.692 a	16.777 b	10.001 c	8.821 b	64.78	-6.61	33.03	33.69	78.70	58 a	37 b	5
Pooled variance	0.022	0.012	0.018	0.014	0.012	0.024	0.53	0.78	0.64	1.35	1.48	3	3	2

[†] NS: not significant at p< 0.05; *, **, and ***, significant at p< 0.05, 0.01, and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different (p<0.05), Tukey's least significant difference test. [§] 'Others' means broken shell, unpeeled, dark color, etc.

Table 2. Fatty acid composition of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Fatty Acids (%)											
	C14:0	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**	**C20:1**	**SFA**	**MUFA**	**PUFA**
ANOVA Test[†]												
Treatment	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Rootstock	NS	**	**	NS	*	*	*	NS	NS	NS	**	NS
Treat. x Rootstock	NS	NS	NS	NS	NS	*	NS	NS	*	NS	*	NS
Tukey's Multiple Range Test[‡]												
Treatment												
T0	0.08	12.49	1.05	1.34	52.78	31.23	0.55	0.12	0.32	14.03	54.15	31.78
T1	0.08	12.38	1.10	1.30	53.23	30.85	0.51	0.14	0.34	13.90	54.67	31.36
T2	0.08	12.29	1.04	1.36	52.90	31.29	0.54	0.13	0.36	13.86	54.30	31.83
Rootstock												
P. Integerrima	0.08	12.17 b	1.03 b	1.29	52.91 ab	31.49 a	0.54 ab	0.13	0.34	13.67	54.88 a	32.03
P. Atlantica	0.08	12.61 a	1.13 a	1.31	52.17 b	31.64 a	0.55 a	0.14	0.34	14.14	53.64 b	32.19
P. Terebinthus	0.07	12.39 ab	1.03 b	1.40	53.88 a	30.24 b	0.50 b	0.12	0.34	13.98	55.25 a	31.74
Treat. x Rootstock												
T0* *P. Integerrima*	0.08	12.39	1.04	1.32	53.59	30.60 b	0.57	0.10	0.29 b	13.89	54.91 b	31.17
T1* *P. Integerrima*	0.08	12.20	1.07	1.27	52.94	31.42 ab	0.52	0.15	0.35 ab	13.70	54.36 bc	31.94
T2* *P. Integerrima*	0.09	11.98	0.99	1.27	52.36	32.25 a	0.54	0.12	0.38 a	13.47	53.74 c	32.78
T0* *P. Atlantica*	0.09	12.60	1.11	1.38	51.56	32.17 a	0.58	0.15	0.36 ab	14.22	53.03 c	32.75
T1* *P. Atlantica*	0.08	12.62	1.16	1.29	53.32	30.46 b	0.53	0.15	0.30 b	14.15	54.79 b	30.99
T2* *P. Atlantica*	0.07	12.59	1.09	1.26	51.63	32.28 a	0.55	0.13	0.35 ab	14.05	53.07 c	32.82
T0* *P. Terebinthus*	0.08	12.52	1.01	1.31	53.42	30.67 b	0.50	0.10	0.31 ab	14.01	54.74 b	31.17
T1* *P. Terebinthus*	0.07	12.33	1.03	1.34	53.31	30.95 b	0.48	0.13	0.36 ab	13.87	54.70 b	31.43
T2* *P. Terebinthus*	0.07	12.31	1.05	1.55	54.70	29.33 b	0.52	0.14	0.34 ab	14.06	56.09 a	29.85
Pooled variance	0.02	0.33	0.08	0.13	1.44	1.45	0.05	0.03	0.04	0.24	1.65	1.48

[†] NS: not significant at $p < 0.05$; * and **, significant at $p < 0.05$ and 0.01, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. Mineral content of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	K	Na	Ca	Mg	Cu	Mn	Fe	Zn
	Macro-elements (g kg^{-1})				Micro-elements (mg kg^{-1})			
ANOVA Test[†]								
Treatment	***	*	**	NS	***	***	NS	NS
Rootstock	NS	NS	NS	***	**	**	***	***
Treat. x Rootstock	*	NS	NS	NS	*	***	NS	NS
Tukey's Multiple Range Test[‡]								
Treatment								
T0	9.373 a	0.264 b	0.980 b	1.105	16.41 c	6.96 a	27.34	21.59
T1	9.512 a	0.279 ab	1.063 a	1.109	22.01 b	7.06 a	30.45	21.85
T2	9.050 b	0.294 a	1.047 a	1.136	28.25 a	5.88 b	30.91	22.99
Rootstock								
P. Integerrima	9.412	0.285	1.022	1.079 b	19.40 b	6.01 b	23.54 b	16.5 b
P. Atlantica	9.360	0.270	1.035	1.128 a	24.23 a	6.72 ab	31.83 a	24.0 a
P. Terebinthus	9.178	0.269	1.056	1.158 a	23.91 a	7.21 a	34.53 a	25.3 a
Treat. x Rootstock								
T0* *P. Integerrima*	9.471 ab	0.263	0.976	1.076	14.95 c	6.52 ab	22.01	17.35
T1* *P. Integerrima*	9.301ab	0.299	1.016	1.102	23.55 b	6.13 ab	25.42	17.33
T2* *P. Integerrima*	8.749 c	0.294	1.077	1.060	19.97 bc	5.31 b	23.20	15.06
T0* *P. Atlantica*	9.596 ab	0.267	0.987	1.135	18.48 bc	6.67 ab	29.10	23.95
T1* *P. Atlantica*	9.502 ab	0.270	1.079	1.093	23.28 b	7.26 a	32.41	22.75
T2* *P. Atlantica*	9.196 bc	0.273	1.041	1.157	31.36 a	6.15 ab	33.98	25.60
T0* *P. Terebinthus*	9.160 bc	0.271	1.005	1.146	17.67 c	7.40 a	34.28	23.65
T1* *P. Terebinthus*	9.746 a	0.260	1.124	1.133	19.28 bc	7.82 a	33.58	26.13
T2* *P. Terebinthus*	9.218 b	0.275	1.045	1.194	35.96 a	6.13 ab	35.73	26.16
Pooled variance	0.206	0.020	0.071	0.088	0.63	0.54	3.42	1.53

[†] NS: not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

Table 4. Total polyphenol content [g gallic acid equivalent (GAE) kg^{-1} dry weigh, dw] and antioxidant activity (mmol Trolox kg^{-1} dw) of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	TPC	DPPH	FRAP	ABTS
	(g GAE kg^{-1} dw)	(mmol Trolox kg^{-1} dw)		
ANOVA Test†				
Treatment	*	***	***	NS
Rootstock	NS	NS	NS	NS
Treatment x Rootstock	*	***	NS	NS
Tukey's Multiple Range Test‡				
Treatment				
T0	1192 b	19.19 a	35.45 a	21.54
T1	1284 a	18.46 a	33.49 ab	22.82
T2	1211 ab	10.64 b	29.63 b	20.98
Rootstock				
P. Integerrima	1242	14.12	32.08	21.75
P. Atlantica	1212	16.86	32.20	21.63
P. Terebinthus	1187	17.31	33.30	21.97
Treat. x Rootstock				
T0* *P. Integerrima*	1212 ab	19.63 a	35.91	21.55
T1* *P. Integerrima*	1287 a	21.79 a	35.85	24.14
T2* *P. Integerrima*	1063 b	9.19 b	26.95	20.15
T0* *P. Atlantica*	1158 b	18.84 a	35.58	21.83
T1* *P. Atlantica*	1249 a	21.77 a	31.32	22.79
T2* *P. Atlantica*	1230 ab	11.31 b	29.62	20.13
T0* *P. Terebinthus*	1204 b	19.09 a	34.01	21.29
T1* *P. Terebinthus*	1255 a	11.83 b	30.19	21.51
T2* *P. Terebinthus*	1267 a	11.44 b	32.08	22.29
Pooled variance	76	2.80	4.64	2.20

†NS: not significant at $p < 0.05$; * and ***, significant at $p < 0.05$ and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

Table 5. Descriptive sensory analysis of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Visual		Flavor							Texture					
	Color	Size	Nutty	Pistachio-ID	Floral	Salty	Sweet	Bitter	Astringent	Aftertaste	Wood	Hardness	Crunchiness	Friability	Adhesiveness
ANOVA Test[†]															
Treatment	*	***	NS	***	NS	NS	NS	NS	NS	**	NS	NS	*	NS	NS
Rootstock	NS	***	***	***	NS	**	*	NS	NS	**	NS	***	***	***	***
Treat. × Rootstock	***	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	***	NS	NS
Tukey's Multiple Range Test[‡]															
Treatment															
T0	3.5 a	7.7 a	7.3	7.3 a	2.9	0.7	2.9	1.0	0.8	7.4 a	5.5	7.0	7.0 b	5.0	6.7
T1	3.2 ab	7.8 a	6.9	6.9 a	1.6	0.5	2.6	1.3	0.8	7.4 a	5.8	7.0	6.7 b	5.5	7.0
T2	2.8 b	5.1 b	7.4	6.2 b	1.7	0.4	2.7	1.1	0.8	6.8 b	5.9	7.8	7.8 a	5.4	6.3
Rootstock															
P. Integerrima	3.2	6.3 b	7.7 a	7.6 a	1.5	0.8 a	2.2 b	1.2	0.8	7.6 a	6.3	8.1 a	7.8 a	6.6 a	5.7 b
P. Atlantica	3.3	7.5 a	7.2 ab	6.2 b	1.8	0.4 b	2.7 ab	1.1	0.8	7.1 ab	5.8	6.1 b	5.9 b	5.7 a	7.0 a
P. Terebinthus	3.1	6.8 b	6.7 b	6.6 b	1.7	0.4 b	3.2 a	0.9	0.8	6.9 b	5.7	7.6 a	7.8 a	4.5 b	7.3 a
Treat. × Rootstock															
T0* *P. Integerrima*	3.8 a	7.0 b	7.9	8.1	1.7	1.4	2.3	1.2	0.7	8.0	6.3	8.4 a	8.2 a	6.4	5.5
T1* *P. Integerrima*	2.9 b	7.2 b	7.2	6.7	1.6	0.7	2.3	1.1	0.7	7.2	6.6	7.2 b	6.6 b	6.0	6.5
T2* *P. Integerrima*	2.9 b	4.5 c	8.0	7.9	1.3	0.4	2.1	1.3	0.8	7.5	6.3	8.8 a	8.6 a	7.4	5.1
T0* *P. Atlantica*	4.1 a	8.0 ab	6.9	6.9	1.8	0.4	3.1	0.8	0.8	7.3	6.6	4.6 c	4.6 c	4.0	7.6
T1* *P. Atlantica*	2.9 b	9.0 a	7.5	6.0	1.6	0.4	2.4	1.6	0.7	6.9	4.0	7.4 b	7.0 b	5.3	6.5
T2* *P. Atlantica*	2.9 b	5.5 c	7.2	5.7	2.0	0.4	2.5	1.0	0.7	7.2	4.1	6.4 b	6.1 b	4.4	7.0
T0* *P. Terebinthus*	2.7 b	8.0 ab	7.0	7.0	1.7	0.4	3.3	0.9	0.9	7.0	4.9	8.0 a	8.0 a	5.5	7.0
T1* *P. Terebinthus*	3.8 a	7.1 b	6.0	5.8	1.6	0.4	3.2	1.0	0.8	6.4	6.1	6.5 b	6.5 b	5.7	8.1
T2* *P. Terebinthus*	2.7 b	5.3 c	7.0	7.1	1.7	0.4	3.1	1.0	0.9	7.4	6.3	8.1 a	8.8 a	5.9	6.7
Pooled variance	0.3	0.8	0.5	0.3	1.2	0.3	0.4	0.3	0.2	0.6	0.8	0.8	0.4	0.9	0.7

[†] NS: not significant at $p < 0.05$; *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

PUBLICATION 4

INFLUENCE OF ROOTSTOCK AND REGULATED DEFICIT IRRIGATION ON PISTACHIO QUALITY

Luis Noguera-Artiaga, Paola Sánchez-Bravo, David Pérez-López, Antoni Szumny, Ángel Calin-Sánchez, Armando Burgos-Hernández, Ángel A. Carbonell-Barrachina

Foods, 2019

(En revisión)

Índice de calidad

Journal Citation Reports (2018)

Área: Food Science & Technology

Factor de impacto: 3.011 (36/135)

Cuartil: Q2

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Journal: Foods
Manuscript ID: foods-643937
Title: Influence of rootstock and regulated deficit irrigation on pistachio quality
Authors: Luis Noguera-Artiaga , Paola Sánchez-Bravo , David Pérez-López , Antoni Szumny , Angel Calin-Sánchez , Armando Burgos-Hernández , Ángel Antonio Carbonell-Barrachina *

Received: 31 October 2019
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INFLUENCE OF ROOTSTOCK AND REGULATED DEFICIT IRRIGATION ON PISTACHIO QUALITY

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Declaration of interest: none

ABSTRACT

Climate change, the increase in world population, and the intensification of urban and industrial activities, will cause a shortage of water for agriculture. This situation requires conscientious studies to manage water deficits without affecting the quality of the crops. In this study, regulated deficit irrigation (RDI) strategies and three rootstocks (P. atlantica, P. integerrima, and P. terebinthus) were applied to pistachio cultivation to study the quality of fruits obtained based on the morphological, functional, aroma, and their sensory properties. Results obtained demonstrated that RDI T1 (during phenological phase II of cultivation the stem water potential was maintained around −1.5 MPa) led to obtain pistachios with same morphological properties, total polyphenol content, antioxidant activity, volatile composition, sensory properties, better profile of fatty acids, and were the favorite ones to international consumers, than pistachios obtained under fully irrigation treatments. On the other hand, when P. integerrima is used pistachios obtained had high weight, less content of sucrose and better functional properties.

KEYWORDS: Antioxidant activity; fatty acid methyl esters; hydroSOS; *Pistacia vera*; pistachio flavor; quality; sensory analysis; total polyphenol content.

1. INTRODUCTION

Countries that border the Mediterranean Sea, South of America, Southern California, Southern Australia and South Africa, there are characterized by partially wet spring and autumn, mostly rainy winters and hot dry summers. To complement water scarcity and avoid water deficits in plants, scarce rainfall must be complemented with irrigation treatments. In addition, climate change, the increase in world population, and the intensification of urban and industrial activities, will cause a shortage of water for agriculture, which is becoming increasingly severe [1]. This situation requires more conscientious studies to manage water deficits without affecting the quality of the crops. These studies should be focus on crops able to withstand deficit irrigation or less water needs without an impact on production and fruit quality [2].

One of the techniques focused on the reduction of water during the cultivation of fruits and vegetables is the regulated deficit irrigation (RDI). RDI consists of the imposition of water deficits in specific phenological stages, which are less sensitive without affect the crop yield or its economic benefits [3,4].

Pistachio (*Pistacia vera*) is considered the only commercially edible nut among the different species in the genus *Pistacia*, has been cultivated for centuries in Mediterranean areas and is considered resistant to drought and salinity [5]. This is based on parameters obtained from crop yields, but the physicochemical, functional and sensory quality of nuts has not been fully characterized. For their vegetative propagation, pistachio trees requires the use of rootstocks, because is a species that is not possible to propagate by cutting and planting due to not enough roots are produced [6]. The main rootstocks used to pistachio cultivation are *P. atlantica* Desf., *P. integerrima* L., *P. terebinthus* L. and *P. vera* L. [7].

Cultivation of pistachio trees has become a very profitable business, because in the last years, their harvesting was fully mechanized, the inputs associated to their cultivation was decreasing, and the prize paid to producers was highly increased [2]. In the future, this trend is expected to increase due to the studies that support the health effects of pistachio consumption [8,9]. It has been proved that the pistachio antioxidant capacity, total phenolic content, monounsaturated and polyunsaturated acids, lutein, phytosterols, and another functional compounds (founded on the pistachio nuts), were related to the anti-inflammatory potential, helping to encourage cardiovascular health, and foster protective effect against colorectal and breast cancer of this nuts [10-13].

For all the above reasons, it is necessary to establish or identify those parameters that allow characterizing the quality of pistachios. In this sense, the main objective of this study was evaluating the quality of pistachio nuts obtained under 3

irrigation treatments and 3 rootstocks, based on their morphological properties, fatty acids content, antioxidant properties, total polyphenol content, volatile composition and their sensory properties.

2. MATERIALS AND METHODS

2.1. Plant material, growing conditions and experimental design

Pistachio nuts from trees (*P. vera*), cultivar "*Kerman*" were collected during 2016 from the experimental orchard "La Entresierra" located at Ciudad Real, Spain (3°56' W, 39° N; altitude 640 m above sea level). This area is characterized by a Mediterranean climate, with an average annual rainfall of 397 mm. The soil is a shallow clay-loam (Petrocalcic Palexeralfs) with a discontinuous petrocalcic horizon located at 0.5 m with a pH about 8.1, low electrical conductivity (0.2 dS m^{-1}), 1.05 % organic matter, 0.12 % N, $17x10^{-4}$ mol kg^{-1} K and a high cation exchange capacity (0.186 mol kg^{-1}).

Eighteen plots were used for this study with a completely randomized factorial design. Each of these plots had 12 trees (2 on the center for the analyses and 10 surrounded them) with same conditions of irrigation and rootstock.

Pistachio trees were grafted over 3 rootstocks: *P. atlantica*, *P. integerrima* and *P. terebinthus,* and, 3 irrigation treatments: T0, in which trees were irrigated to ensure non-limiting water conditions in the soil (100 % ET_C of the previous week); T1, in which irrigation was suppressed (during phase II) until pistachio trees reached a stem water potential (SWP) below −1.5 MPa; and T2 with same irrigation protocol as T1 but with a SWP threshold of −2.0 MPa.

Water relations were characterized according to Memmi [14].

Pistachio nuts were collected from the field, and after peeled and dried (convection oven with hot air at 60 °C until the moisture content of 5 %), were immediately vacuum packed and were posted to Universidad Miguel Hernández de Elche, Orihuela (Alicante, Spain). Once there, samples were kept at 4-5 °C until analysis.

2.2. Morphological analysis

Twenty-five pistachio nuts from each treatment were randomly selected and used to determine the size, weight, and color. In addition, pistachios were classified by their condition of split open, non-split open and others (uncommercial: broken shell, unpeeled, dark color, etc.). For the determination of the size, the length, width and height of the edible part from each pistachio were measured using a digital caliper (model 500-197-20 150 mm; Mitutoyo Corp., Aurora, IL, USA). In case of

weight, the whole nut, shell and edible part were weighed (model AG204 scale; Mettler Toledo, Barcelona, Spain) with a precision of 0.1 mg. For the color, these 25 pistachios were ground (Taurus Aromatic Ver II; Taurus Group, Barcelona, Spain) and placed in Petri dishes (100x15 mm). The color was measured using a colorimeter (model CR-300, Minolta, Osaka, Japan) with an illuminant D65 and an observer of 10 °. Color data were provided as CIE*L*a*b** coordinates.

2.3. Fatty acids

For the determination of fatty acid methyl esters (FAME), the organic layer of the extracts was identified and quantified using a gas chromatograph, Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detect (GCMS QP-5050A). The chromatograph was equipped with a column (polar) Suprawax 280, 100 % polyethylene glycol (Teknokroma S. Co. Ltd., Barcelona, Spain; 30 m x 0.25 m x 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 1.1 mL min^{-1} (split ratio 1:10). The temperature on the injector was 230 ºC; on the detector, the temperature was 260 °C. The oven program and the identification of peaks were carry out following the method described by Carbonell-Barrachina [7].

2.4. Determination of sugars and organic acids

Sugars and organic acids were identified and quantified according to Hernández [15], with some modifications. One gram of sample was diluted in 5 mL of phosphate buffer (pH 7.8), homogenized by Ultra-Turrax™ (IKA L004640) for 1 min, and centrifuged at 15000 x g for 10 min. Finally, samples were filtered through a 0.45 µm Millipore filter.

For the determination of the content of sugars and organic acids on samples, the high-performance liquid chromatography a Hewlett-Packard series 1100 (Hewlett-Packard, Wilmington, DE, USA) were used. The elution buffer consisted of 0.1 % phosphoric acid with a flow rate of 0.5 mL min^{-1}.

Sugars and organic acids were isolated using a Supelco column (Supelcogel TM C-610H column 30 cm × 7.8 mm, Supelco, Inc., Bellefonte, PA, USA) and a precolumn Supelguard (5 cm × 4.6 mm; Supelco), and the absorbance was measured at 210 nm using a diode-array detector (DAD). Standards of sugars (glucose, fructose, sucrose, raffinose, maltitol, and sorbitol) and organic acids (oxalic, citric, tartaric, malic, quinic, shikimic, succinic and fumaric) were obtained from Sigma (Poole, UK). Calibration curves were used for the quantification of sugars and organic acids, showing good linearity (R2 =0.999). Results for both organic acids and sugars were expressed as concentrations g L^{-1} of dry weight (dw).

2.5. Total polyphenol content and antioxidant activity

For the total polyphenol content (TPC) determination and the antioxidant activity of the pistachios affected by rootstock and irrigation treatments, a methanol extract was prepared. Ten milliliters of MeOH/water (80:20, v/v) plus 1% HCl were added to 0.5 g of crushed pistachio. Then, the mixture was sonicated at 20 °C for 15 min and left at 4 °C for 24 h. After that, the extract was sonicated again for 15 min and centrifuged at 10,000 x g during 10 min.

TPC was quantified using the Folin-Ciocalteu colorimetric method described by Gao [16], with some modifications. To 0.1 mL of the methanolic extract were added 0.2 mL of Folin-Ciocalteu reagent and 2 mL of distilled water; then, samples were incubated for 3 min (room temperature). After that, 1 mL of 20 % sodium carbonate was added and incubated again for 1 hour. The absorbance was determined by measurement at 765 nm using an UV-visible spectrophotometer (Helios Gamma model, UVG 1002E). Quantification was carried out according to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), g kg^{-1} (dw).

For the analysis of the antioxidant activity were used the methods $ABTS^{+}$ [17], FRAP [18]. and DPPH* [19]. Ten-µL of the supernatant of the methanolic extract was mixed with 990 µL of reagent $ABTS^{+}$ or FRAP. After reaction (10 min) the absorbance was measured at 734 nm for ABTS+ and 593 nm in case of FRAP method. For the DPPH method, 10 µL of the supernatant was mixed with 40 µL of MeOH and 950 µL of $DPPH^{\bullet}$ solution. Then the mixture was shaken, placed under dark conditions (15 min), and its absorbance was determined at 515 nm. The results obtained on the analysis of the antioxidant activity of pistachio samples, were expressed as mmol Trolox kg^{-1} dw.

2.6. Volatile compounds

The extraction of the volatile compounds of the samples of pistachios was carried out using the headspace solid-phase micro-extraction (HS-SPME) method. A sample of 1 g of ground pistachios was placed on a 50 mL vial, with a magnetic bar, and closed with an aluminum layer (foil). After equilibration time, 5 min at 45 ºC, a 30/50 µm fiber (SUPELCO) covered by DVB/CAR/PDMS (Divinylbenzene / Carboxen / Polydimethylsiloxane) was exposed to the vial headspace at 45 ºC, with continuous agitation (500 rpm) in a magnetic stirrer (IKA C-MAG HS 4, IKA - Werke GmbH & Co. KG, Staufen, Germany). After 25 min of exposure, fiber was put in a gas chromatograph to the analysis.

The isolation and identification of the volatile compounds previously extracted by HS-SPME were performed using a Saturn 2000 Varian Chrompack gas chromatograph (Varian, Inc., CA, USA) with an HP-5 column (5% Phenyl Methylpolysiloxane) 30 m x 0.53 mm ID x 1.0 µm (Agilent, CA, USA). A mass spectrometer equipped with an ion analyzer was set to 1508 V for all analyzes, and an electronic voltage factor to 1350 V. The analysis was carried out from 39 to 400 m / z, with an electronic impact (EI) of 70 eV, in 1 scan / s mode. Helium was used as carrier gas at a flow rate 1.0 mL / min and with a split ratio of 1:20. The injector and detector temperature were 200 and 300 ºC, respectively. The oven temperature started at 40 ºC and, after 3 min, was increased by 5 ºC min^{-1} up to 110 ºC. Then, the temperature was increased by 20 ºC min^{-1} up to 270 ºC. The total analysis program lasts 25 min.

The volatile compounds were identified using three analytical methods: Kovats Index (KI), GC-MS retention index (original chemical compound), and mass spectrum (original chemical compound and collection of the NIST05 and Adams 2012 spectrum library). The retention indexes were used by standard of aliphatic hydrocarbons, soluble in methanol, in the range from C-5 to C-23. For the identification and determination of volatile compounds, the MS Workstation (Version 6.5, 2005 Varian) and MestReNova (Version 9.0.1, 2014) programs were used.

2.7. Sensory analysis

The sensory analysis of samples was focused only on the analysis of the pistachios obtained under different irrigation treatments, in order to minimize the number of samples and, thus, maintain the panelists concentration to the maximum. Based on previous results [7], pistachio nuts obtained by *P. atlantica* rootstock were used on the sensorial study.

To obtain information about the sensory properties of pistachios, a sensory evaluation with consumer panel were carry out in 3 countries: Mexico, Poland, and Spain. At least 60 consumers were recruited in each country. Consumers had to complete a screener stating their age, gender, and allergies or diet restrictions. Consumers were asked about nut consumption frequency and willingness to taste pistachios. Consumers who stated that they were 18–70 years old, had no diet restrictions or allergies, ate any kind of nut at least once per week and were willing to taste pistachios were recruited for testing. In the specific case of Poland, to confirm no major misinterpretations took place during the translation process, the ballots, screeners and demographic questionnaires were translated from Spanish to Polish, and then back to Spanish.

Ten pistachio nuts were served, to each panelist, in odor-free disposable 60 mL covered plastic cups, coded using three-digit numbers, and at room temperature. Between samples, to clean the panelists' palate were used unsalted crackers and drinking water. Natural illumination was used during the test, and testing room was at 20±2 ºC.

Consumers responded using a 9-point hedonic scale, where 9= like extremely and 1 = dislike extremely. Consumers were then asked to indicate their order of preference for the samples, and mark the reasons of their election between all of the attributes under study (size, peel, color, pistachio-ID, toasted, sweet, sour, aftertaste, oiliness, hardness, crunchiness, friability and adhesiveness). Then, consumers were asked about their global satisfaction degree for the samples under evaluation and for their intent to purchase.

2.8. Statistical analysis

The data presented in this study are the mean values of 3 replicates and was subjected to two-way analysis of variance (ANOVA). Then, data were subjected to Tukey's multiple-range test to compare the means. Percentage data were transformed by Arcosen function before statistical analysis. Differences were considered statistically significant at $p<0.05$. All statistical analyses were done using XLSTAT software (version 2014.1).

3. RESULTS AND DISCUSSION

3.1. Morphological analysis

On the analysis of split-open and non-split open pistachios, **Table 1**, no statistically differences were observed among samples obtained under different irrigation treatments, being the mean values 54 % and 42 %, respectively. In case of effect of rootstock, P. terebinthus had the highest number of split pistachios (60 %) and, as could it be otherwise, the less number of non-split open pistachios (35 %). *P. atlantica* and *P. integerrima* were statistically related.

The moderated reduction of water during phenological phase II of pistachios (T1) had no effect on the weight and size of nuts obtained (**Table 1**). On the other hand, high reduction of water during these phase (T2), led to pistachios with less weight of their edible nut (T0 = 0.692 g and T2 = 0.673 g).

Regarding rootstocks, no statistically differences were founded on the weight of whole nut and shell, and on the length and height of pistachio nuts. However, samples of *P. integerrima* and *P. atlantica* had the highest weight of the edible nut (**Table 1**).

Color of the samples had statistically significant differences in the parameters L* and a*, in case of irrigation and rootstock. These differences were minimum, and some authors have concluded that differences smaller than 2 units, how is the case (**Table 1**), were imperceptible for the human eye [20,21].

Similar results were previously reported by Carbonell-Barrachina [7], who showed that neither rootstocks nor RDI treatments did significantly affect the morphological parameters of pistachio nuts.

3.2. Fatty acids

Nine fatty acids (FAMEs) were identified by GC-MS in pistachio samples (**Table 2**): 2 were polyunsaturated (PUFAs) [α-linolenic acid (C18:3) and linoleic acid (C18:2)]; 3 monounsaturated (MUFAs) [eicosenoic acid (C20:1), oleic acid (C18:1), and palmitoleic acid (C16:1)]; and, 4 saturated (SFAs) [arachidic acid (C20:0), stearic acid (C18:0), palmitic acid (C16:0), and myristic acid (C14:0)]. The three main FAMEs founded in pistachio samples were *C18:1 (~53 % of the total), C18:2* (~31 %), and *C16:0* (~12 %), while the three ones founded in less amount were *C14:0* (~0.07 %), *C20:0* (~0.16 %), and *C20:1* (0.36 %).

Pistachios obtained under moderate RDI, T1 had higher content of oleic acid and less content of α-linolenic than control (T0) and T2 treatment (**Table 2**). In case of rootstocks, no statistically differences were observed on the fatty acid composition except on the content of α-linolenic acid, where *P. atlantica* had the lowest values of concentration (**Table 2**). According to the interaction between the two factors studied, rootstock and irrigation, pistachios obtained by *P. integerrima* and T1 had the higher content of oleic acid (**Table 2**).

The application of treatment T1 had influence on the content of unsaturated fatty acid composition of pistachios, increasing the content of MUFA, and decreasing the content of PUFA. Use of different rootstocks had no effect on the composition of the SFA, MUFA or PUFA.

In previous studies, carried out under same conditions, Carbonell-Barrachina [7] obtained that regulated deficit irrigation (moderate) increased the content of linoleic acid, while in this study the one that has been increased has been the oleic acid. Acar [22] reported that main fatty acids founded in pistachio were oleic, linoleic and palmitic acids as has been obtained in this study.

3.3. Sugars and organic acids

In the study of the sugars and organic acids on the composition of pistachios obtained under RDI and rootstocks, 3 sugars (maltitol, raffinose and sucrose) and 3

organic acids (fumaric, oxalic, and shikimic) were identified and quantified (**Table 3**).

Samples obtained under treatment of irrigation T1 had less concentration of fumaric acid (0.287 g L^{-1}) than pistachios control (T0 = 0.315 g L^{-1}), while nuts of T2 (0.287 g L^{-1}) were statistically related with T0 and T1. In the rest of organic acids and sugars, no statistically differences were found between pistachios obtained under different irrigation treatments (**Table 3**).

In case of rootstocks, *P. integerrima* led to pistachios with less concentration of sucrose (19.770 g L^{-1}) versus *P. terebinthus* and *P. atlantica* (22.505 and 24.977 g L^{-1}, respectively). In the analysis of organic acids*, P. integerrima* was the rootstock that produce higher concentrations of the 3 acids studied, while *P. terebinthus* had less amount of oxalic and fumaric acids (**Table 3**).

According to interaction of the 2 factors studied, pistachios obtained under P. integerrima and irrigation T1 had the lowest concentration of sucrose, while rootstock P. atlantica and T2 led to pistachios with the highest concentration (**Table 3**).

Similar results were obtained by Lipan [24] in almonds affected by RDI (sucrose, main sugar, did not affected by irrigation treatments).

3.4. Antioxidant activity (AA) and total polyphenol content (TPC)

Results obtained on the study of AA and TPC are shown in **Table 4**. In general, pistachios had higher functional potential based on their high total polyphenol content (~1350 g GAE kg^{-1}, dw) and their antioxidant activity (~22 mmol Trolox kg^{-1}, dw, on the 3 methods studied). Similar results of antioxidant activity and content of polyphenols were found in previous studies with pistachios affected by different irrigation treatments [4,7,29].

The application of moderate regulated irrigation treatments (T1) on the cultivation of pistachios had no statistically incidence in the antioxidant activity and total phenolic compounds of nuts obtained. On the contrary, when the water restriction was severe (T2) the AA of pistachios were reduced (with DPPH and FRAP methods). Under situations of moderate water stress, plants redistribute the CO_2 to the formation of secondary metabolites as a physiological response for the removal the free radicals formed; while under high stress, this CO_2 is dedicated to primary metabolism [23,24].

In case of the study of rootstocks, *P. integerrima* led to obtain pistachio nuts with higher concentrations of TPC and AA than rootstocks *P. atlantica* and *P. terebinthus* (**Table 4**).

Similar results of antioxidant activity and content of polyphenols were found in previous studies with pistachios affected by different irrigation treatments [4,7,25].

3.5. Volatile compounds

Thirty-one compounds were identified in the volatile profile of pistachios under study (**Table S1**) and were characterized according their sensory descriptors. The three most abundant compounds were α-pinene (~35 %), limonene (~14 %), and β-myrcene (~11 %), and irrigation and rootstocks demonstrated that affect statistically to the content of all of them (**Table 6**). This results agreed with those obtained by Hojjati [26] and Penci [27] in previous studies, who also found that the same main compounds as predominant in the aromatic profile of pistachio and its essential oil.

Treatment of irrigation T1 had not statistically significative differences on the volatile composition of pistachios nuts with respect to control (T0). On the other hand, pistachios obtained under RDI T2 had less amounts of α-pinene, dodecane and tridecane, and high content of β-myrcene and limonene than T0. In case of pistachios obtained under rootstocks, *P. integerrima* had the highest content of α-pinene (main volatile compound of pistachios); *P. atlantica* led to nuts with high content of β-myrcene, dodecane and tridecane; and, *P. terebinthus* had the high content of limonene.

In previous studies, Carbonell-Barrachina [7] demonstrated that regulated deficit irrigation treatments led to pistachio nuts with similar or even better amounts of the main volatile compounds. These results are in concordance with obtained in this study, in order to both studies demonstrated that the application of RDI technics had no negative effect on the volatile composition of pistachio nuts.

Based on the study of the interaction between the two factors studied, if we want to obtain pistachios with high content of α-pinene, it is necessary to use the rootstock *P. integerrima* and the irrigation treatments T0 or T1. On the other hand, if we want to obtain pistachios with more citrus aroma (more limonene) the combination of the *P. terebinthus* rootstock and irrigation treatment T1 will be the most successful.

According to results obtained, the main volatile compounds found in the volatile profile of pistachios α-pinene, limonene, and β-myrcene (compounds sensory related with descriptors of woody, citrus and fruity, respectively), can be used as a control tool to study the quality of the aroma of this nut.

3.6. Sensory analysis

Around 200 consumers from Mexico, Spain and Poland (at least 60 in each country) participated in the pistachio affective sensory analysis. In Mexico, a 68 % of panelist were women, 37 % in Poland, and 55 % in case of Spain. Of the total number of consumers, 35 % were between 18-25 years old, 32 % were between 26-

35, 15 % between 36-45 years old, 17 % between 46-55 years old, and 2 % were older than 55 years old.

The irrigation treatments significantly affected 3 of the thirteen sensory attributes under analysis (**Table 6**): *pistachio-ID*, *oiliness*, and *overall*. Pistachios obtained under RDI T1 obtained higher intensities of *pistachio-ID* (6.7) than control and T2 (6.4 and 6.4). This result was observed in each of the countries under study (**Table 6**).

In the attribute *oiliness*, T1 and T0 obtained scores of 6.0 while T2 obtained 5.8. Mexican consumers rated the score of this attribute with less intensity than Polish and Spanish consumers, like in case of *hardness* and *crunchiness*. The most consumed dried fruit in Mexico is peanut, so it is possible that consumers in this country expect a little more *oiliness*, *hardness* and *crunchiness* in pistachios samples, hoping to find a texture similar to that of fried peanuts.

In addition, in case of *overall*, the attribute that define the final opinion of consumers about the overall quality of sample, treatment T1 obtained the highest score (6.7), while T0 get 6.3 and T2 was statistically related with them (score of 6.5). If we analyze the results in function of the country of consumers, this same result was observed in Mexico and Spain. However, Polish consumers did not appreciate significantly differences on the overall quality between samples obtained by the three irrigations treatments (**Table 6**).

When we force the consumers to choose (among the three samples studied) which was his favorite sample, pistachios obtained under RDI T1 were the most likes in each of the countries (in case of Poland, no statistically differences were observed between T1 and T0). On the other hand, sample that least liked to consumers in each country was T2 (**Figure 1**). Same results were observed when asked to consumers about their willingness to pay the samples under study. Consumers mentioned that the main reasons for selecting the preferred sample were the pistachio flavor (~83 %), crunchiness (~65 %), aftertaste (~45 %) and hardness (~30 %) (**Figure 1**).

Similar results were observed in previous studies. Carbonell-Barrachina [7] obtained that the purchase choice of international pistachio consumers based on the sensory attributes is due to pistachio flavor, saltiness, crunchiness and toasted flavor. Although, Noguera-Artiaga [28] exposed that international consumers preferred intense crunchy but low salty nuts.

4. CONCLUSIONS

The results obtained in this study demonstrated that apply moderate deficit irrigation during pistachio cultivation (T1) led to obtain pistachios with same

morphological properties, total polyphenol content, antioxidant activity, volatile composition and sensory properties than pistachios obtained under fully irrigation treatments (T0). Moreover, T1 led to pistachios with better profile of fatty acids and were the favorite sample for international consumers. On the contrary, when the RDI is severe (T2), pistachio nuts had less antioxidant activity, less polyphenols content, and were the least favorite for the consumers. In case of pistachios obtained under different rootstocks, P. integerrima led to pistachio nuts with high weight, less content of sucrose and better functional properties, than P. atlantica and P. terebinthus.

These results demonstrated that is possible saving water during pistachio cultivation, with the consequent less environmental and economic cost, and obtain pistachio nuts with same or even better quality attributes.

ACKNOWLEDGEMENTS

Authors want to thank to Departamento de Investigación y Posgrado en Alimentos of the University of Sonora (Mexico) and the Faculty of Biotechnology and Food Science of the Wrocław University of Environmental and Life Sciences (Poland), for their collaboration and helpness in this study. The authors are grateful to the projects AGL2013-45922-C2-1-R and AGL2013-45922-C2-2-R (Ministerio de Economía y Competitividad, Spain). Luis Noguera-Artiaga was funded by an FPU grant from the Spanish government (FPU014/01874).

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Table 1. Weight, size, and nature of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Weight (g)			Size (mm)			Number *per* 100 units			Color coordinates				
	Whole	Edible Nut	Shell	Length	Height	Width	Split open	Non-split open	Others[§]	L	a*	b*	C*	Hue
ANOVA test[†]														
Irrigation	NS	***	NS	NS	***	***	NS	NS	NS	***	NS	NS	NS	***
Rootstock	NS	***	NS	NS	NS	***	***	***	NS	***	***	NS	NS	***
Irrigation*Rootstock	NS	***	NS	NS	***	***	*	*	NS	***	***	NS	NS	***
Tukey's multiple range test[‡]														
Irrigation														
T0	2.062	0.692 a	1.370	17.10	10.78 a	9.48 ab	55	42	4	67.74 a	-5.93	32.60	33.15	100.28 a
T1	2.051	0.695 a	1.356	17.23	10.74 a	9.56 a	53	43	4	66.37 b	-5.63	32.98	33.47	99.69 ab
T2	2.009	0.673 b	1.335	17.06	10.49 b	9.35 b	54	42	4	66.44 b	-5.54	32.67	33.16	99.61 b
Rootstock														
P. Atlantica	2.036	0.686 ab	1.350	17.16	10.62	9.43 b	50 b	46 a	4	66.95 ab	-5.39 a	32.86	33.31	99.27 b
P. Integerrima	2.047	0.695 a	1.352	17.18	10.68	9.61 a	52 b	46 a	2	67.32 a	-6.34 b	32.51	33.13	101.02 a
P. Terebinthus	2.038	0.679 b	1.359	17.05	10.71	9.36 b	60 a	35 b	5	66.29 b	-5.38 a	32.88	33.33	99.29 b
Irrigation*Rootstock														
T0* *P. Atlantica*	2.061	0.691 ab	1.369	17.16	10.71 abc	9.37 bc	50 b	46 a	4	66.68 bc	-5.79 ab	33.16	33.68	99.87 ab
T1* *P. Atlantica*	2.029	0.688 ab	1.340	17.28	10.73 ab	9.41 abc	49 b	47 a	4	66.90 bc	-5.61 ab	32.79	33.27	99.69 ab
T2* *P. Atlantica*	2.018	0.678 ab	1.339	17.04	10.42 c	9.47 abc	51 b	44 a	5	67.26 b	-4.75 a	32.61	32.97	98.24 b
T0* *P. Integerrima*	2.062	0.702 a	1.360	17.15	10.91 a	9.67 ab	53 b	45 a	4	68.75 a	-6.59 b	32.56	33.23	101.43 a
T1* *P. Integerrima*	2.061	0.702 a	1.358	17.16	10.61 abc	9.74 a	51 b	46 a	4	66.49 c	-5.84 ab	32.64	33.16	100.15 a
T2* *P. Integerrima*	2.017	0.681 ab	1.336	17.24	10.54 bc	9.41 abc	51 b	47 a	3	66.74 bc	-6.58 b	32.34	33.01	101.48 a
T0* *P. Terebinthus*	2.062	0.682 ab	1.379	16.99	10.75 ab	9.37 bc	62 a	34 b	4	67.79 ab	-5.41 ab	32.07	32.53	99.52 ab
T1* *P. Terebinthus*	2.062	0.693 ab	1.368	17.25	10.88 a	9.54 ab	60 a	36 b	4	65.74 cd	-5.44 ab	33.51	33.96	99.23 ab
T2* *P. Terebinthus*	1.990	0.661 b	1.329	16.91	10.51 bc	9.17 c	59 a	36 b	5	65.34 d	-5.29 ab	33.07	33.51	99.11 ab

[†] NS: not significant at p< 0.05; * and ***: significant at p< 0.05 and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different (p<0.05), Tukey's least significant difference test. [§] 'Others' means unpeeled, broken shell, dark color, etc.

Table 2. Fatty acid composition of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	Fatty Acids (%)											
	C14:0	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C20:0**	**C20:1**	**SFA**	**MUFA**	**PUFA**
ANOVA Test[†]												
Irrigation	NS	NS	NS	NS	**	NS	**	NS	NS	NS	**	**
Rootstock	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
Treat. x Rootstock	NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS	NS
Tukey's Multiple Range Test[‡]												
Irrigation												
T0	0.07	12.03	1.28	1.28	51.94 b	32.18	0.73 a	0.16	0.32	13.55	53.54 b	32.92 a
T1	0.07	11.60	1.21	1.52	54.95 a	29.54	0.62 b	0.16	0.33	13.34	56.49 a	30.16 b
T2	0.07	11.86	1.19	1.32	52.05 b	32.39	0.67 ab	0.15	0.30	13.39	53.54 b	33.06 a
Rootstock												
P. Atlantica	0.07	11.90	1.20	1.38	53.34	31.00	0.63 b	0.18	0.31	13.52	54.85	31.63
P. Integerrima	0.08	11.63	1.19	1.37	52.95	31.63	0.67 ab	0.15	0.33	13.22	54.47	32.31
P. Terebinthus	0.07	11.96	1.29	1.36	52.66	31.48	0.73 a	0.15	0.31	13.54	54.26	32.21
Irrigation*Rootstock												
T0* *P. Atlantica*	0.07	12.13	1.26	1.28	52.09 b	32.01 ab	0.64	0.20	0.31	13.69	53.66	32.65
T1* *P. Atlantica*	0.07	11.59	1.17	1.29	52.95 b	31.87 ab	0.61	0.17	0.28	13.11	54.41	32.48
T2* *P. Atlantica*	0.06	11.97	1.17	1.58	54.98 ab	29.12 ab	0.63	0.16	0.33	13.77	56.47	29.76
T0* *P. Integerrima*	0.08	11.95	1.30	1.28	50.96 b	33.18 ab	0.78	0.14	0.33	13.44	52.60	33.96
T1* *P. Integerrima*	0.07	11.27	1.10	1.69	58.44 a	26.33 b	0.58	0.19	0.33	13.22	59.87	26.92
T2* *P. Integerrima*	0.08	11.66	1.18	1.16	49.45 b	35.38 a	0.66	0.11	0.33	13.01	50.95	36.05
T0* *P. Terebinthus*	0.07	12.01	1.28	1.28	52.76 b	31.36 ab	0.78	0.15	0.31	13.51	54.35	32.14
T1* *P. Terebinthus*	0.06	11.93	1.35	1.58	53.47 b	30.41 ab	0.68	0.14	0.37	13.71	55.20	31.09
T2* *P. Terebinthus*	0.08	11.93	1.23	1.23	51.73 b	32.65 ab	0.73	0.17	0.25	13.40	53.22	33.38

[†] NS: not significant at p< 0.05; * and **, significant at p< 0.05 and 0.01, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different (p< 0.05), Tukey's least significant difference test. SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids.

Table 3. Sugars (g L^{-1}) and organic acids (g L^{-1}) on pistachios obtained under regulated deficit irrigation and different rootstocks.

Factor	Sugars (g L^{-1} dw)			Organic acids (g L^{-1} dw)		
	Raffinose	**Sucrose**	**Maltitol**	**Oxalic**	**Shikimic**	**Fumaric**
ANOVA test[†]						
Irrigation	NS	NS	NS	NS	NS	***
Rootstock	NS	***	NS	***	***	***
Irrigation*Rootstock	NS	***	NS	NS	NS	***
Tukey's multiple range test[‡]						
Irrigation						
T0	9.793	22.831	4.111	0.156	0.535	0.315 a
T1	9.077	20.449	4.848	0.127	0.581	0.287 b
T2	10.351	23.972	5.796	0.148	0.555	0.296 ab
Rootstock						
P. Atlantica	10.345	24.977 a	5.772	0.165 a	0.467 b	0.260 b
P. Integerrima	9.517	19.770 b	5.257	0.151 a	0.737 a	0.328 a
P. Terebinthus	9.359	22.505 a	3.727	0.116 b	0.468 b	0.309 a
Irrigation*Rootstock						
T0* *P. Atlantica*	9.091	23.692 ab	4.441	0.189	0.456	0.273 bc
T1* *P. Atlantica*	8.963	21.574 ab	4.447	0.145	0.418	0.266 bc
T2* *P. Atlantica*	12.982	29.666 a	8.427	0.160	0.527	0.241 c
T0* *P. Integerrima*	10.418	20.981 ab	3.911	0.137	0.701	0.339 a
T1* *P. Integerrima*	9.237	18.240 b	6.503	0.140	0.832	0.299 ab
T2* *P. Integerrima*	8.895	20.090 ab	5.355	0.175	0.678	0.347 a
T0* *P. Terebinthus*	9.868	23.821 ab	3.982	0.141	0.448	0.332 a
T1* *P. Terebinthus*	9.032	21.534 ab	3.595	0.096	0.494	0.295 abc
T2* *P. Terebinthus*	9.176	22.160 ab	3.605	0.112	0.461	0.300 ab

[†] NS: not significant at $p < 0.05$; ***, significant at $p < 0.001$. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p < 0.05$), Tukey's least significant difference test. dw: dry weight.

Table 4. Total polyphenol content [mg gallic acid equivalents (GAE) kg^{-1} dry weigh, dw] and antioxidant activity (mmol Trolox kg^{-1} dw) of pistachios as affected by deficit irrigation treatment and rootstock.

Factor	TPC	DPPH	FRAP	ABTS
	(mg GAE kg^{-1} dw)	(mmol Trolox kg^{-1} dw)		
ANOVA test[†]				
Irrigation	***	***	***	NS
Rootstock	***	***	***	***
Irrigation*Rootstock	***	***	***	***
Tukey's multiple range test[‡]				
Irrigation				
T0	1390 ab	21.70 a	23.89 a	23.5
T1	1409 a	20.50 ab	24.45 a	23.3
T2	1297 b	18.77 b	19.66 b	22.0
Rootstock				
P. Atlantica	1310 b	19.02 b	20.08 b	21.53 b
P. Integerrima	1522 a	22.09 a	25.47 a	28.08 a
P. Terebinthus	1265 b	19.87 b	22.44 ab	19.07 c
Irrigation*Rootstock				
T0* *P. Atlantica*	1294 bcd	20.06 ab	22.07 abc	22.78 bc
T1* *P. Atlantica*	1450 abc	19.51 ab	21.80 abc	22.85 bc
T2* *P. Atlantica*	1184 d	17.48 b	16.37 c	18.95 c
T0* *P. Integerrima*	1615 a	24.28 a	26.57 ab	29.01 a
T1* *P. Integerrima*	1460 ab	22.53 ab	30.02 a	28.26 a
T2* *P. Integerrima*	1489 ab	19.46 ab	19.82 bc	26.96 ab
T0* *P. Terebinthus*	1260 bcd	20.76 ab	23.03 abc	18.56 c
T1* *P. Terebinthus*	1317 bcd	19.47 ab	21.51 abc	18.71 c
T2* *P. Terebinthus*	1216 cd	19.37 ab	22.77 abc	19.93 c

[†] NS: not significant at $p < 0.05$; ***: significant at $p < 0.001$. [‡] Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

Table 6. Relative content (%) of volatile compounds on pistachios affected by regulated deficit irrigation and rootstocks *P. atlantica* (AT), *P. integerrima* (IN), and *P. terebinthus* (TE).

Compound	ANOVA test†			Irrigation (%)			Rootstock (%)			Irrigation x Rootstock (%)								
	Irrig.	Root.	Irrig. x Root.	T0	T1	T2	AT	IN	TE	AT*T0	AT*T1	AT*T2	IN*T0	IN*T1	IN*T2	TE*T0	TE*T1	TE*T2
Acetic acid	NS	NS	NS	0.46	0.32	0.30	0.33	0.36	0.39	0.33	0.26	0.39	0.59	0.17	0.32	0.45	0.53	0.19
Ethyl acetate	NS	NS	NS	1.04	0.39	0.46	0.69	0.75	0.45	0.85	0.63	0.59	1.48	0.20	0.58	0.79	0.35	0.21
Pentanone	NS	NS	NS	0.25	0.20	0.17	0.19	0.21	0.22	0.20	0.18	0.19	0.33	0.13	0.16	0.21	0.28	0.18
1-Methyl-1H-pyrrole	NS	NS	NS	3.52	4.57	2.82	2.72	2.92	5.26	1.95	3.84	2.39	3.04	3.28	2.44	5.58	6.58	3.62
1-Pentanol	NS	NS	NS	0.92	0.55	0.43	0.67	0.47	0.76	0.90	0.72	0.39	0.67	0.27	0.45	1.18	0.65	0.46
(Z)-3-Octene	**	NS	**	0.26 b‡	0.20 b	0.50 a	0.35	0.27	0.35	0.18 b	0.15 b	0.71 a	0.27 ab	0.13 b	0.39 ab	0.31 ab	0.33 ab	0.41 ab
Hexanal	NS	NS	NS	1.38	0.70	0.94	0.98	0.74	1.30	1.17	0.84	0.91	0.80	0.39	1.03	2.16	0.86	0.88
2-Octene	NS	NS	NS	0.18	0.20	0.27	0.22	0.18	0.24	0.17	0.17	0.31	0.15	0.15	0.24	0.22	0.27	0.24
1-Hexanol	NS	NS	NS	4.39	2.63	2.64	3.98	2.70	2.97	5.75	3.66	2.55	2.86	1.98	3.27	4.56	2.26	2.09
(E)-4-Nonene	NS	NS	**	0.21	0.21	0.30	0.27	0.20	0.24	0.18 b	0.20 ab	0.42 a	0.19 ab	0.14 b	0.26 ab	0.24 ab	0.27 ab	0.21 ab
(Z)-4-Nonene	NS	NS	NS	0.22	0.18	0.22	0.22	0.15	0.24	0.20	0.18	0.28	0.15	0.11	0.19	0.31	0.24	0.18
Nonane	NS	NS	NS	0.25	0.23	0.27	0.29	0.21	0.24	0.30	0.23	0.35	0.20	0.17	0.26	0.26	0.29	0.19
α-Pinene	**	**	**	36.90 a	35.08 a	33.54 b	30.19 b	42.41 a	32.92 b	31.29 c	33.13 c	26.15 d	49.27 a	46.25 a	31.71 c	30.14 c	25.86 d	42.76 b
2-Pentanol	NS	NS	NS	0.37	0.40	0.60	0.56	0.41	0.41	0.23	0.47	0.98	0.43	0.24	0.55	0.45	0.50	0.26
1-Decene	NS	NS	NS	0.83	0.40	0.47	0.75	0.36	0.60	1.10	0.53	0.62	0.47	0.30	0.31	0.94	0.38	0.47
Sabinene	NS	NS	NS	0.48	0.40	0.40	0.38	0.42	0.48	0.43	0.32	0.38	0.54	0.47	0.26	0.47	0.41	0.57
3-Decene	NS	NS	NS	0.62	0.35	0.49	0.51	0.34	0.61	0.44	0.47	0.61	0.41	0.16	0.43	1.00	0.41	0.42
β-Myrcene	**	**	NS	8.39 b	9.58 b	14.89 a	13.21 a	9.79 b	9.86 b	6.77	12.66	20.22	8.16	6.12	15.09	10.26	9.97	9.37
Decane	NS	NS	NS	2.07	2.53	2.74	2.92	2.26	2.16	2.13	3.27	3.34	2.09	1.96	2.73	1.99	2.35	2.14
3-Carene	NS	NS	NS	0.24	0.39	0.39	0.29	0.34	0.39	0.34	0.26	0.26	0.19	0.52	0.31	0.18	0.39	0.61
Limonene	***	**	**	12.19 b	13.72 b	15.01 a	11.19 b	12.24 b	17.49 a	14.87 c	8.11 de	10.60 d	7.91 e	12.73 cd	16.07 bc	13.80 c	20.32 a	18.36 b
(E)-3-Hexenol	NS	NS	NS	0.92	0.94	1.06	1.17	0.82	0.92	0.84	1.23	1.43	0.81	0.64	1.01	1.10	0.94	0.72
2-Octen-1-ol	NS	NS	NS	0.74	0.97	1.11	1.21	0.85	0.75	0.76	1.32	1.54	0.77	0.67	1.12	0.67	0.93	0.65
2-Methyl-decane	NS	NS	NS	0.68	0.45	0.49	0.68	0.46	0.48	0.83	0.60	0.61	0.48	0.31	0.58	0.72	0.45	0.26
Terpinolene	NS	NS	NS	0.66	0.95	0.76	0.81	0.84	0.72	1.06	0.79	0.57	0.40	1.43	0.70	0.52	0.62	1.02
Undecane	NS	NS	NS	8.59	9.40	8.77	9.53	8.77	8.46	9.87	7.73	11.00	7.67	9.17	9.47	8.22	11.32	5.84
2-Nonen-1-ol	NS	NS	NS	1.70	0.90	0.94	1.46	1.04	1.04	2.51	0.81	1.06	1.03	1.19	0.90	1.57	0.69	0.88
1-Nonanol	NS	NS	NS	1.54	1.20	1.01	1.72	0.90	1.13	2.34	1.68	1.13	0.75	1.02	0.94	1.55	0.90	0.95
Dodecane	**	**	**	6.25 ab	8.02 a	5.29 b	8.32 a	5.71 b	5.54 b	7.73 ab	10.51 a	6.71 ab	5.19 b	6.45 ab	5.46 ab	5.83 ab	7.09 ab	3.70 b
Decanal	NS	NS	NS	0.41	0.51	0.46	0.54	0.45	0.39	0.50	0.56	0.56	0.42	0.49	0.45	0.31	0.46	0.38
Tridecane	**	**	**	2.85 ab	3.45 a	2.26 b	3.66 a	2.44 b	2.47 b	3.75 ab	4.50 a	2.73 ab	2.26 ab	2.76 ab	2.28 ab	2.54 ab	3.09 ab	1.76 b

† NS: not significant at p< 0.05; ** and ***: significant at p< 0.01 and 0.001, respectively. ‡ Values (mean of 3 replications) followed by the same letter, within the same volatile compound and factor, were not significantly different (p<0.05), Tukey's least significant difference test.

Table 7. Sensorial affective test of hydroSOS pistachios with consumers of Spain, Mexico, and Poland.

Factor	Size	Peel	Color	Pistachio ID	Toasted	Sweet	Sour	Aftertaste	Oiliness	Hardness	Crunchiness	Friability	Adhesiveness	Overall
ANOVA test[†]														
Irrigation	NS	NS	NS	**	NS	NS	NS	NS	*	NS	NS	NS	NS	**
Country	NS	NS	NS	NS	NS	NS	NS	NS	*	*	**	NS	NS	NS
Country*Irrigation	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS	NS	*
Tukey's multiple range test[‡]														
Irrigation														
T0	6.6	6.8	6.4	6.4 b	6.4	5.8	5.8	6.3	6.0 a	6.5	6.4	6.0	5.7	6.3 b
T1	6.7	6.9	6.5	6.7 a	6.3	6.0	5.6	6.1	6.0 a	6.6	6.4	6.0	5.7	6.7 a
T2	6.5	6.7	6.3	6.4 b	6.5	5.8	5.9	5.9	5.8 b	6.5	6.5	6.2	5.4	6.5 ab
Country														
Mexico	6.5	6.8	6.3	6.3	6.5	5.8	5.8	5.8	5.6 b	6.2 b	6.0 b	5.9	5.5	6.4
Poland	6.9	6.9	6.5	6.7	6.4	5.9	5.8	6.3	6.0 a	6.8 a	6.7 a	6.2	5.9	6.7
Spain	6.5	6.6	6.3	6.5	6.2	5.9	5.6	6.3	6.2 a	6.6 ab	6.7 a	6.1	5.6	6.5
*Country*Irrigation*														
Mexico*T0	6.7	6.9	6.4	6.2 c	6.4	5.9	6.0	6.1	5.6 b	6.1	6.1	5.7	5.5	6.0 b
Mexico*T1	6.5	6.9	6.4	6.6 ab	6.5	5.8	5.5	5.9	5.7 b	6.4	5.9	6.0	5.7	6.6 a
Mexico*T2	6.2	6.6	6.2	6.2 c	6.7	5.7	5.8	5.4	5.6 b	6.2	5.9	6.1	5.1	6.4 ab
Poland*T0	6.7	6.9	6.5	6.6 ab	6.5	5.7	5.8	6.2	6.2 a	6.8	6.5	6.2	5.9	6.9 a
Poland*T1	6.9	6.9	6.4	6.8 a	6.3	6.3	5.8	6.1	6.1 a	6.8	6.7	6.1	6.0	6.7 a
Poland*T2	6.9	6.9	6.7	6.7 a	6.5	5.8	6.0	6.4	5.8 ab	6.8	6.8	6.2	5.7	6.6 a
Spain*T0	6.4	6.6	6.3	6.4 b	6.2	5.8	5.5	6.5	6.3 a	6.7	6.5	6.0	5.8	6.1 b
Spain*T1	6.8	6.8	6.5	6.8 a	6.0	6.0	5.6	6.3	6.2 a	6.5	6.7	5.9	5.5	6.8 a
Spain*T2	6.3	6.5	6.1	6.3 bc	6.4	5.8	5.8	6.0	6.0 ab	6.5	6.9	6.3	5.4	6.5 ab

[†] NS: not significant at p< 0.05; ** and ***: significant at p< 0.01 and 0.001, respectively. [‡] Values (mean of 3 replications) followed by the same letter, within the same sensory attribute and factor, were not significantly different (p<0.05), Tukey's least significant difference test.

Table S1. Identification and sensory descriptors of volatile compounds on pistachios affected by regulated deficit irrigation and rootstock

#	Compound	Rt (min)	KI (exp.)	KI (lit.)	Sensory descriptor
1	Acetic acid	2.21	603	605	Vinegar
2	Ethyl acetate	2.44	601	612	Pineapple; Anise
3	Pentanone	3.52	702	705	Fruity; Cheese; Chocolate
4	1-Methyl-1H-pyrrole	4.38	748	743	Woody; Smoky; Herbal
5	1-Pentanol	5.03	774	765	Sweet; Vanilla; Fusel
6	(Z)-3-Octene	5.56	792	790	nf
7	Hexanal	5.85	801	800	Fatty; Green
8	2-Octene	6.26	818	815	nf
9	1-Hexanol	7.95	874	868	Green; Herbaceous; Woody
10	(E)-4-Nonene	8.51	890	890	nf
11	(Z)-4-Nonene	8.64	893	893	nf
12	Nonane	8.90	900	900	Gasoline
13	*α*-Pinene	9.99	938	937	Woody
14	2-Pentanol	10.30	948	950	Oily; Green
15	1-Decene	11.21	975	981	nf
16	Sabinene	11.38	980	975	Woody; Pine; Spicy
17	3-Decene	11.71	989	988	nf
18	*β*-Myrcene	11.84	992	991	Fruity; Herbaceous; Sweet
19	Decane	12.15	1000	1000	Alkane
20	3-Carene	12.48	1012	1011	Lemon
21	Limonene	13.06	1032	1031	Citrus; Sweet
22	(E)-3-Hexenol	13.29	1040	1038	Green
23	2-Octen-1-ol	13.86	1057	1061	Citrus; Fruity; Green; Vegetable
24	2-Methyl-decane	14.19	1068	1064	nf
25	Terpinolene	14.95	1090	1089	Plastic
26	Undecane	15.31	1100	1100	Alkane
27	2-Nonen-1-ol	15.43	1106	1105	Melon; Waxy
28	1-Nonanol	17.39	1180	1176	Citrus; Rose
29	Dodecane	18.00	1201	1200	Alkane
30	Decanal	18.11	1205	1206	Floral; Citrus; Sweet; Waxy
31	Tridecane	19.48	1301	1300	Alkane

Rt = Retention time; KI (exp.)= Kovat's index experimental; KI (lit.) = Kovat's index literature; nf = not found.

Capítulo 4

Estudio de la calidad funcional de pistachos hidrosostenibles

PUBLICATION 5

PHENOLIC AND TRITERPENOID COMPOSITION AND INHIBITION OF A-AMYLASE OF PISTACHIO KERNELS (*PISTACIA VERA* L.) AS AFFECTED BY ROOTSTOCK AND IRRIGATION TREATMENT

Luis Noguera-Artiaga, David Pérez-López, Armando Burgos-Hernández, Aneta Wojdyło, Ángel A. Carbonell-Barrachina.

Food Chemistry, 261, 240-245, 2018

DOI: https://doi.org/10.1016/j.foodchem.2018.04.033

Índice de calidad

Journal Citation Reports (2018)

Área: Food Science & Technology

Factor de impacto: 5.399 (7/135)

Cuartil: Q1

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Running title: Phenolics in hydroSOS pistachios

ABSTRACT

The current water scarcity forces farmers to adopt new irrigation strategies to save water without jeopardizing the fruit yield and quality. In this study, the influence of 3 regulated deficit irrigation (RDI) treatments and 3 rootstocks on the functional quality of pistachios were studied. The functional parameters studied included, polyphenols, triterpenoids, and inhibition of α-amylase. The results showed that *P. terebinthus* and *P. atlantica* rootstocks led to pistachio kernels with higher contents of polyphenols and triterpenoids (mainly betulinic acid with 111 and 102 μg g^{-1}, respectively) than pistachios obtained using *P. integerrima* rootstock (81 μg g^{-1}). On the other hand, the use of moderate RDI (T1 treatment) increased the total content of polyphenols (~10 %), quercetin-*O*-galloyl-hexoside (~15 %), keampferol-3-*O*-glucoside (~19 %), and polymeric procyanidins (~20 %), as compared to the control trees, resulting in pistachios with a better functional profile, lower economic cost and with a lesser environmental impact.

KEYWORDS: polymeric procyanidins; hydroSOS; regulated deficit irrigation; functional properties; LC-PDA-MS-Qtof.

1. INTRODUCTION

Pistachio (*Pistacia vera* L.) is a popular and delicious nut and due to a highly favorable taste, nutritional value, and health promoting composition is widely consumed worldwide as a snack, either fresh or roasted and salted. Furthermore, pistachio is normally used as an ingredient in cakes, confectionery products, biscuits, candies, ice creams, chocolates, sausages, and sauces (Hojjati, Noguera-Artiaga, Wojdyło, & Carbonell-Barrachina, 2015; Kahyaoglu, 2008).

A pistachio nut consists of a fleshy hull (epicarp and mesocarp) surrounding the nut shell, which encloses the nutmeat. As the nutmeat grows, it fills the shell and at the end, its growth exceeds the shell size, and cracks the shell. In general, the hull remains intact during this entire process, protecting the nutmeat from insects and pathogens (Grace, Esposito, Timmers, Xiong, Yousef, Komarnytsky, et al., 2016).

The world food production heavily depends on water availability, and this resource is getting more and more scarce, especially for agricultural purposes. Thus, it is absolutely necessary to improve farming efficiency through the use of drought-resistant species and/or through techniques that reduce the volume of irrigation water (Grant, 2012). Regulated deficit irrigation (RDI) is a system of managing water supply by imposing some water deficits at specific phenological stages, which have been found to be less sensitive, with no or low reduction of yield and consequently economic benefits (Gijón, Gimenez, Perez-López, Guerrero, Couceiro, & Moriana, 2011). Fruits and vegetables cultivated under RDI are called hydrosustainable or hydroSOS products and have a solid identity based on (i) an increase of their secondary metabolites, that improves their functionality and quality of these products, and (ii) environmental respect because they optimize the use of water (Noguera-Artiaga, Lipan, Vázquez-Araújo, Barber, Pérez-López, & Carbonell-Barrachina, 2016).

During the phenological development of pistachio nut there are differentiated 3 different stages (Carbonell-Barrachina, Memmi, Noguera-Artiaga, del Carmen Gijón-López, Ciapa, & Pérez-López, 2015; Goldhamer, 1995):

- *stage I* starts at the beginning of nut growth and finishes when maximum nut size is reached;
- *stage II* is the period in which the shell hardens; and,
- *stage III* is the period in which the edible part grow.

It is well known that stage II in pistachio nut development is the less sensitive to water stress (Gijón, Gimenez, Perez-López, Guerrero, Couceiro, & Moriana, 2011;

Goldhamer, 1995); thus, the deficit irrigation strategies used in the current experiment were based on restricting irrigation water only during stage II.

Pistachio cultivation requires the use of rootstocks, and grafting is the only form of vegetative propagation. Thus, it is essential to study the influence of the rootstock on the nut yield, and the functionality and quality of the edible kernels. The main pistachio rootstocks are *Pistacia vera* L. in Iran, *P. integerrima* L. and a hybrid between *P. integerrima* Steward ex Brandis and *P. atlantica* Desf. (UCB-I) in the USA, and *P. terebinthus* L. in the Mediterranean basin (Carbonell-Barrachina, Memmi, Noguera-Artiaga, del Carmen Gijón-López, Ciapa, & Pérez-López, 2015; Moriana, Memmi, Centeno, Martín-Palomo, Corell, Torrecillas, et al., 2018).

The beneficial effects of pistachio consumption on decreasing the levels of the main risk factors of cardiovascular disease (CVD), such as lipids, endothelial function, inflammation, blood pressure, and oxidative status are well known (Bisignano, Filocamo, Faulks, & Mandalari, 2013). Phytochemicals previously identified from pistachios include fatty acids, phytosterols, and polyphenols; all of them have been identified as protective agents against CVD and even cancer (Liu, 2004). Furthermore, the bioactive constituents of nuts have cardioprotective, antiobesity, anticancer, and antioxidant properties (Casas-Agustench, López-Uriarte, Ros, Bulló, & Salas-Salvadó, 2011; Martínez-González & Bes-Rastrollo, 2011; Urpi-Sarda, Casas, Chiva-Blanch, Romero-Mamani, Valderas-Martínez, Arranz, et al., 2012; Yang, Fletcher, & Reilly, 2009).

To the best of our knowledge, there have been no reports in the literature comparing the bioactive compounds (polyphenols and triterpenoids) of pistachio kernels obtained after grafting on different rootstocks and being cultivated under different irrigation treatments. Thus, the aim of this work was to evaluate the profile of bioactive compounds (polyphenol identification and quantification, polymeric procyanidins, triterpenoids content and inhibitory effects of α-amylase) of pistachio kernels obtained using 3 rootstocks and 3 RDI treatments. The results will help in deciding the best rootstock for Kerman pistachio trees and whether the application of regulated deficit irrigation have any potential benefits for edible pistachio kernels regarding their content of bioactive compounds.

2. MATERIALS AND METHODS

2.1 Chemicals

Methanol, oleanolic, betulinic, and ursolic acids, 3,5-dinitrosalicylic acid, dipotassium hydrogen, phloroglucinol, p-nitrophenyl-α-D-glucopyranoside, orthophosphate dihydrogen, potassium sodium tartrate tetrahydrate, sodium phosphate

monobasic, and α-amylase from porcine pancreas (type VI-8) were purchased from Sigma-Aldrich (Steinheim, Germany). (+)-Catechin, and (−)-epicatechin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, procyanidin B1 and B2, eriodictyol-3-O-glucoside and quercetin and keampferol-3-*O*-glucoside were purchased from Extrasynthese (Lyon Nord, France). Both, ascorbic acid and acetonitrile for ultra-pressure liquid chromatography (UPLC, gradient grade), were purchased from Merck (Darmstadt, Germany). UPLC grade water, prepared by HLP SMART 1000s system (Hydrolab, Gdańsk, Poland), sample was filtrated through a 0.20 μm membrane filter (Millex Samplicity® Filters Membrane) immediately before use.

2.2 Plant material and experimental design

Pistachio trees, cultivar Kerman, were grown during 2013 and 2014 in an area with a Mediterranean climate (experimental farm "El Chaparrillo", Ciudad Real (Spain), 3º56' W and 39º0' N, altitude 640 m above sea level), with an average annual rainfall of 397 mm, mostly distributed outside a four-month summer drought period. The soil can be classified as shallow clay-loam (*Petrocalcic Palexeralfs*) of 0.5 m depth, and a discontinuous *petrocalcic* horizon of around 0.5 m. Tree spacing was set at 7 × 6 m (238 trees ha^{-1}). Male trees of the cultivar Peter were used and distributed evenly throughout the field, in a proportion of 10 %.

The study was conceived using a factorial design with two replicates and with two factors: (i) type of rootstock and (ii) irrigation treatment. Pistachio trees (*Pistacia vera*) were grafted over 3 different rootstocks*: P. terebinthus* L. (TE)*, P. integerrima* L. (INT), and *P. atlantica* Desf (AT). Three irrigation treatments were evaluated: (i) T0 or control, in which trees were irrigated with enough water volume to completely avoid hydric stress; (ii) T1, in which irrigation was suppressed until trees reached a stem water potential (SWP) below -1.5 MPA during phase II, then, irrigation was managed to keep SWP below this threshold but as near to it as possible; and, (iii) T2, in which the same strategy as in T1 was used, but with a SWP threshold of -2.0 MPa. In both deficit irrigation treatments (T1 and T2), pistachio trees were rehydrated when phase III started. The irrigation protocol for stressed treatments was derived from the methodology proposed by Moriana, Pérez-López, Prieto, Ramírez-Santa-Pau, and Pérez-Rodriguez (2012) for olive trees and later adapted to pistachio trees by Memmi, Gijón, Couceiro, and Pérez-López (2016).

2.3 Polyphenols extraction

The extraction of polyphenols was conducted as described previously by Wojdyło, Oszmiański, and Bielicki (2013) in quinces, and El-Zaeddi, Calín-Sánchez, Nowicka, Martínez-Tomé, Noguera-Artiaga, Burló, et al. (2017) in herbs. Briefly, grounded pistachio

(~0.6 g) was weighed, 5 mL of 30 % of aqueous methanol (v/v) with 1 % of ascorbic acid and 3 mL of hexane were added, and the suspension was stirred, sonicated for 15 min in a ultrasonic bath JP Selecta S.A, model 3000512 (Barcelona, Spain) with constant frequency (40 kHz), left for 24 h at room temperature in darkness, and, then, centrifuged for 10 min (20.878×*g* at 4 ºC); finally, supernatants were analyzed within the same day. The extraction procedure for the analysis of α-amylase activity was the same previously described for polyphenols but using a different solvent, 80 % of aqueous methanol (v/v) with 1 % of HCl (v/v).

2.4 Identification and quantification of polyphenols by LC-PDA-MS-QTof (liquid *chromatography photodiode array quadrupole time-off flight mass spectrometry)*

The samples were analyzed by using an Acquity UPLC, ultra performance liquid chromatography, system (Waters, Milford, MA), and a Micromass QTof spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative mode were used for the identification in pistachio extracts of polyphenols. The UPLC system was used for the separation of individual polyphenols using a UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm, Waters Corp.; Milford, USA) at 30 °C. The flow rate was 0.45 mL min^{-1} and 10 µL of the samples were injected, and only 15 min were needed to complete the elution of the sample. Solvent A (2.5 % formic acid, v/v) and solvent B (100 % acetonitrile) were the mobile phase. The elution process was as follows: (i) 0 - 1 min, isocratic elution with 99 % A; (ii) linear gradient was used until 12 min, lowering A to 0 %; and, (iii) from 12.5 to 13.5 min, the initial composition (99 % A) was used, and was held constant to re-equilibrate the column. Full scan from m/z 100 to 1500 was used for the analysis of the samples. The internal reference compound (leucine encephalin) was introduced via the LockSpray channel and the lock mass correction was ±1.000 for the mass window. All QTof-MS chromatograms are presented as the base peak intensity (BPI) chromatograms and scaled to 12.400 counts per second (cps) (=100 %). The eluent was passed to the electrospray source with the following conditions: capillary voltage of 2500 V, cone voltage of 30 V, source temperature of 130 °C, desolvation temperature of 350 °C and desolvation gas (nitrogen) flow rate 300 L h^{-1}.

Polyphenolic compounds were monitored at the following wavelengths, 520 nm (anthocyanins), 360 nm (flavonols and flavanones), 320 nm (phenolic acids), and 280 nm (flavan-3-ols). Quantification was done by injecting standard solutions of known concentrations according to Wojdyło, Nowicka, Carbonell-Barrachina, and Hernández (2016). This analysis was run in triplicate, and results were expressed as mg *per* 100 g dry matter (dm) of pistachio kernel.

2.5 Analysis of triterpenoids by the LC-MS-PDA (liquid chromatography-mass spectrometry-photodiode array)

Sample extraction was performed as described by Wojdyło, Nowicka, Carbonell-Barrachina, and Hernández (2016) in figs, and Kolniak-Ostek (2016) in pears. The identification and quantification of oleanolic, betulinic, and ursolic acids were performed using an ACQUITY Ultra Performance LC system with binary solvent manager (Waters Corp.), a UPLC BEH C18 column (1.7 μm, 2.1 × 150 mm, Waters Corp.), a QTof mass spectrometer (Waters Corp.) equipped with an ESI source operating in negative mode, and methanol–acetonitrile (15:85, v/v) as an elution solvent. The compounds were monitored at 210 nm; the m/z for betulinic, ursolic, and oleanolic acids were 455.3452, 455.3365, 455.3496, respectively, and their retention times were 6.80, 7.50, and 7.85 min, respectively. This analysis was run in triplicate, and results were expressed as μg g^{-1} dm.

2.6 Polymeric procyanidins (PP)

The protocol described previously by Kennedy and Jones (2001) and later modified by Wojdyło, Carbonell-Barrachina, Legua, and Hernández (2016) was used to produce the phloroglucinolysis of the pistachio samples. These samples were treated with a solution of HCl/MeOH/phloroglucinol/ascorbic acid (solution of 0.3 M HCl in MeOH containing 75 g L^{-1} phloroglucinol and 10 g L^{-1} ascorbic acid) and after 30 min, 1.2 mL of aqueous sodium acetate was added to stop the reaction. Polymeric proanthocyanidins were analyzed by UPLC–FL (ACQUITY UPLC™; Waters Corporation; Milford, USA), and using wavelengths of excitation/emission of 278/330 nm. Sample was injected into a BEH RP Shield C18 column (2.1 mm × 50 mm; 1.7 μm) with precolumn (Waters Corp., Milford, MA, USA) operated at 15 °C; the injection volume was 5 μL, and flow rate was 0.45 mL min^{-1}. The elution solvents were 2.5 % aqueous acetic acid (v/v) (A) and 100 % acetonitrile (B). Samples were eluted using the following solvent program: 0–0.6 min, 2 % B; 0.6–2.17 min, 2–3 % B; 2.17–3.22 min, 3–10 % B; 3.22–5.00 min, 10–15 % B; 5.00–6.00 min, 100 % B; and finally, washing and reconditioning of the column for next 1.50 min. Procyanidin B1 and B2 were used as reference compounds and were subjected to phloroglucinolysis degradation as described above, and the conversion rate of (−)-epicatechin and (+)-catechin was calculated. The mean degree of polymerization (DP) was obtained by calculating the molar ratio of both extension and terminal flavan-3-ol units to terminal units (Wojdyło, Oszmiański, & Bielicki, 2013). This analysis was run in triplicate and results were expressed as mg *per* 100 g dm of pistachio nut.

2.7 α-Amylase inhibition

The α-amylase inhibition was measured according to the procedure described initially by González-Muñoz, Quesille-Villalobos, Fuentealba, Shetty, and Gálvez Ranilla

(2013), and later modified by Nowicka, Wojdyło, and Samoticha (2016). The α-amylase inhibitory effect of pistachio was assayed by measuring the absorbance at 540 nm using a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). This analysis was run in triplicate and results were expressed as IC_{50} (mg of dried nut mL^{-1}).

2.8 Statistical Analyses

The data was subjected to two-way analysis of variance (ANOVA) and later to Tukey's multiple-range test to compare the means. Differences were considered statistically significant at $p<0.05$. All statistical analyses were done using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD).

3. RESULTS AND DISCUSSION

3.1. Qualitative analysis of phenolic compounds

Seventeen compounds were identified in the methanolic extract of pistachio samples using LC-PDA-MS-QTof: 8 flavonols, 4 flavan-3-ols, 2 anthocyanins, 2 phenolic acids, and 1 flavanone (**Table 1**). The phenolic compounds were identified according to their retention times, molecular masses, fragmentation patterns, characteristic spectra, and literature references (Fabani, Luna, Baroni, Monferran, Ighani, Tapia, et al., 2013; Grace, et al., 2016; Gültekin-Özgüven, Davarci, Pasli, Demir, & Özçelik, 2015; Mandalari, Bisignano, Filocamo, Chessa, Sarò, Torre, et al., 2013; Regueiro, Sánchez-González, Vallverdú-Queralt, Simal-Gándara, Lamuela-Raventós, & Izquierdo-Pulido, 2014). The combination of LC-PDA-MS-QTof analysis and electrospray ionization (ESI) mass spectrometry allowed efficient identification of all polyphenols found in the pistachio samples under analysis.

Flavonoids have a C6–C3–C6 general structural backbone in which the two C6 units (rings A and B) are of phenolic nature. Due to the hydroxylation pattern and variations in the chromane ring (ring C), flavonoids can be further divided into sub-groups, such as anthocyanins, flavan-3-ols, flavones, flavanones, and flavonols (Tsao, 2010). In case of the pistachio under analysis, the predominant sub-group was flavan-3-ols being the main compound identified as (+)-catechin (peak at 2.65 min), with a $(M-H)^-$ ion at *m/z* 289.0727 and MS/MS fragments at *m/z* 245, 205, and 179. Also it was possible to identify (-)-epicatechin with the same $(M-H)^-$ ion as (+)-catechin, but different retention time (peak at 3.55 min). Apart from these compounds, two procyanidins dimers were identified (peaks at 3.22 and 9.00 min) with *m/z* 577 and a characteristic fragmentation pattern for a monomer of a negatively charged molecular ion $[M-H]^-$ at *m/z* 289. In the case of flavonols, there were two types of derivatives, with a fragment at *m/z* 285 and 301, characteristic

for kaempferol and quercetin, respectively (**Table 1**). Furthermore, two anthocyanins with a fragment at *m/z* 287 were found, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoside (peaks at 4.21 and 4.27 min, respectively). Finally, there was one flavanone, eriodictyol-3-*O*-glucoside ($[M-H]^-$ at *m/z* 449), and one sugar derivative, deoxydihexoside, at retention time 2.87 min and with a characteristic fragmentation pattern $[M-H]^-$ at m/z 309 (Kitrytė, Kraujalienė, Šulniūtė, Pukalskas, & Venskutonis, 2017).

3.2. Quantification of phenolic compounds

The main phenolic compound found in pistachio samples was (+)-catechin with values ranging 97 and 143 mg $(100\ g)^{-1}$ dm, followed by (-)-epicatechin with values from 25.5 to 42.6 mg $(100\ g)^{-1}$ dm. These two compounds have been also reported as the predominant phenolic compound in the skin and kernels of pistachios by others researchers (Fabani, et al., 2013; Gültekin-Özgüven, Davarci, Pasli, Demir, & Özçelik, 2015; Mandalari, et al., 2013; Tomaino, Martorana, Arcoraci, Monteleone, Giovinazzo, & Saija, 2010).

The use of different rootstocks proved to have a significant effect on the individual contents of caffeic acid hexoside and keampferol-*O*-dihexoside; with the rootstock *P. terebinthus* getting the highest contents 9.18 and 1.91 mg $(100\ g)^{-1}$ dm, respectively (**Table 2**). However, no significant differences were found in the rest of phenolic compounds.

The use of regulated deficit irrigation in pistachios caused a decrease in the concentration of the main compound, (+)-catechin; its content was directly related to the amount of water reduced during cultivation, with T0 pistachios having the highest concentration [143 mg $(100\ g)^{-1}$ dm], followed by T1 nuts [119 mg $(100\ g)^{-1}$ dm], and T2 [97.6 mg $(100\ g)^{-1}$ dm]. On the other hand, application of moderate regulated deficit irrigation (T1) had a significant effect on the concentration of keampferol-3-*O*-glucoside and quercetin-*O*-galloyl-hexoside, increasing their contents from 1.6 (T0 and T2) to 1.8 mg $(100\ g)^{-1}$ dm (T1), and from ~6.8 (T0 and T2) to 8.1 mg $(100\ g)^{-1}$ dm (T1), respectively (**Table 2**).

The concentration of polymeric procyanidins (PP) in pistachio samples ranged from 323 to 443 mg $(100\ g)^{-1}$ dm and statistically significant differences were found for both of the factors under study, rootstock and irrigation treatment (**Table 2**). Pistachios obtained with *P. terebinthus* and *P. atlantica* rootstocks [443 and 415 mg $(100\ g)^{-1}$ dm, respectively] had higher PP content than those obtained using the *P. integerrima* rootstock [323 mg $(100\ g)^{-1}$ dm]. In case of pistachios obtained by different irrigation treatments, the application of RDI treatments caused a significant increase of the PP contents, increasing

from 352 mg (100 g)$^{-1}$ dm in control samples up to 427 and 404 mg (100 g)$^{-1}$ dm in T1 and T2 samples, respectively (**Table 2**).

If the total content of phenolic compounds is considered, the pistachios obtained with *P. terebinthus* and *P. atlantica* rootstocks had higher concentration [680 and 636 mg (100 g)$^{-1}$, respectively] than those obtained using *P. integerrima* rootstock [554 mg (100 g)$^{-1}$ dm]. On the other hand, the application of a severe RDI treatment, T2, did not have a significant incidence as compared to the control treatment; [T0= 603 mg (100 g)$^{-1}$ dm; T2 = 604 mg (100 g)$^{-1}$ dm], while the application of a moderate deficit irrigation (T1) increased the total concentration of phenolic compounds up to 664 mg (100 g)$^{-1}$ dm (about 10 % of the total content) (**Table 2**).

This increase in the total content of phenolic compounds and especially the increase of PP in pistachio kernels obtained under moderate water deficit (T1) agreed with results obtained by Sun, Liang, Shan, Viernstein, and Unger (2011) and Guo, Duan, Tang, Qian, Zhu, Qian, et al. (2011) for jujube fruits, and Galindo, Calín-Sánchez, Griñán, Rodríguez, Cruz, Girón, et al. (2017) for pomegranates. They postulated that polyphenolic compounds, especially PP, increased under harsh growing conditions, i.e. water deficit. Therefore, in plants under water deficit (T1 and T2), when carbohydrates exceed the amount used for growth concentrations, the excess of CO_2 assimilated could increase the biosynthesis of carbon-based secondary metabolites (Horner, 1990). Moreover, the increase in the procyanidin content through a water stress effect could also be related with the fact that water deficit can lead to an increase in the levels of free phenylalanine (Chalker-Scott & Fuchigami, 1989), a precursor in the PP synthesis, and an increase in L-phenylalanine ammonia lyase (PAL) activity and, probably PAL synthesis (Chalker-Scott & Fuchigami, 1989; Jess Tovar, Paz Romero, Girona, & Jos Motilva, 2002).

3.3. Quantification of triterpenoids in pistachio kernels

Triterpenoids are known for their antioxidant, anti-inflammatory, and anticancer properties (Claude, Morin, Lafosse, & Andre, 2004). **Table 3** shows the results obtained after quantification of triterpenoids in pistachio as affected by rootstock and irrigation treatment. The detected compounds were identified as betulinic, oleanolic, and ursolic acids based on MS profiles with the fragmentation pathways, UV spectra, and the retention times of authentic standards.

In pistachio samples, the average total of triterpenoids content was ~644 μg g^{-1} dm and it was not significantly affected by neither the rootstock nor the irrigation treatment. Even if the individual contents of each triterpenoid are studied, no significant effect of the irrigation treatment was found. In this way, the mean values for the contents of the three

triterpenoids identified were 98, 60, and 486 μg g^{-1} dm for betulinic, oleanolic, and ursolic acids, respectively. Thus, the predominant compound in this chemical family in pistachio kernels was the ursolic acid.

In case of pistachios obtained under different rootstocks, *P. terebinthus* and *P. Atlantica* had the highest content of betulinic acid (111 and 102 μg g^{-1}, respectively) while *P. integerrima* had the lowest one (81 μg g^{-1}). In the other two studied triterpenoids (oleanolic and ursolic acid), the use of different rootstocks had no significant effect on their concentration (**Table 3**).

3.4. α-Amylase activity in pistachio kernels

Dietary carbohydrates are hydrolyzed in human body by pancreatic α-amylase, one of the enzymes responsible for the breakdown of oligosaccharides and disaccharides into absorbable monosaccharides. Inhibition of that enzyme, by controlling glucose absorption, may be effective in the regulation of type 2 diabetes (Gironés-Vilaplana, Baenas, Villaño, Speisky, García-Viguera, & Moreno, 2014; Wojdyło, Nowicka, Carbonell-Barrachina, & Hernández, 2016). In this study, the α-amylase inhibition was measured in pistachios obtained using different rootstocks and irrigation treatments, and the results are presented as IC_{50} (mg of dried nut mL^{-1}) (**Table 4**). No significant effect was found in pistachios obtained using different rootstocks; however, pistachio samples obtained under RDI (T1 and T2) had significantly higher values of inhibition (IC_{50}= 26.4 and 25.2, respectively) than control nuts (IC_{50}= 19.7). Consequently, results showed that the application of RDI treatments did not eliminate the α-amylase inhibitory activity in pistachio. The α-amylase inhibitory activity in pistachios might be related to their higher phenolic contents (**Table 2**), as triterpenoids have not being proved to have any significant effect on this enzyme; in addition, this effect could be linked to the antioxidant capacity of the phenolic compounds. Recent studies suggested that fruits with high concentration of procyanidins, such as the case of pistachios, are effective inhibitors of α-amylase activity by the formation of enzyme-tannin complexes, which prevented the enzyme from interacting with the starch (Boath, Stewart, & McDougall, 2012; Nowicka, Wojdyło, & Samoticha, 2016). This inhibitory effect on α-amylase demonstrated that pistachio nuts could possess possible beneficial effects in the treatment of diabetes, which must be further confirmed by *in vivo* studies.

To the best of our knowledge, the α–amylase inhibitory activity of pistachios, obtained under different irrigation treatments, has not been previously reported elsewhere. Therefore, the data presented on this study could be assumed as the first report in the literature stating this point.

4. CONCLUSIONS

This was the first study establishing quantitatively the phenolic profiles, triterpenoid compounds, and α-amylase inhibition in pistachio kernels as affected by rootstock and regulated irrigation treatments. After comparing the results obtained using 3 rootstocks (*P. atlantica*, *P. integerrima*, and *P. terebinthus*), it has been demonstrated that the use of the *P. terebinthus* and *P. atlantica* rootstocks led to pistachio kernels with higher functional quality, by increasing the contents of betulinic acid and total polyphenols as compared to the fruits obtained with *P. integerrima* rootstock. On the other hand, reducing the irrigation water volume (regulated deficit irrigation) during the cultivation of pistachio (stage II, shell hardening) has been shown to have a significant impact on the functionality of pistachios. The application of a moderate water reduction treatment (T1) increased the total polyphenol content of the samples, as well as the contents of quercetin-*O*-galloyl-hexoside, keampferol-3-*O*-glucoside, and polymeric procyanidins. When the water reduction was severe (T2), the concentration of polymeric procyanidins was also increased, but the total polyphenolic content was not significantly affected. In case of the α-amylase inhibition, the application of RDI treatments does not eliminate inhibitory activity in pistachio. Therefore, the final conclusion of this study was that the application of a T1 treatment led to pistachios with a better functional profile than the control ones besides reducing the water consumption during its cultivation; this fact is essential because it is linked to a lower economic cost and environmental impact by saving water.

ACKNOWLEDGEMENTS

The authors are grateful to the projects AGL2013-45922-C2-1-R and AGL2013-45922-C2-2-R (Ministerio de Economía y Competitividad, Spain). Luis Noguera-Artiaga was funded by an FPU grant from the Spanish government (FPU014/01874).

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Table 1. Identification of phenolic compounds found in pistachios using LC-MS-QTof/PDA.

Compounds	Retention time (min)	λ_{max} (nm)	MS $[M-H]^-$ (*m/z*)	MS/MS (*m/z*)
(+)-Catechin	2.65	278	289.0727	
Deoxydihexoside	2.87	280	309.1236	
Procyanidin dimer	3.22	280	577.1375	289.0727
(-)-Epicatechin	3.55	275	289.0727	
Caffeic acid hexoside	3.84	270	465.1021	325.0945/303.0502/285.0395
Cyanidin-3-*O*-galactoside	4.21	278/525	447.0887	287.0313
Cyanidin-3-*O*-glucoside	4.27	278/517	447.0887	287.0245
Eriodictyol-3-*O*-glucoside	4.81	285/350	449.1078	389.1755/339.0400/287.0612
Quercetin-*O*-galloyl-hexoside	6.05	270/352	615.2206	365.1801/301.0255
Keampferol-3-*O*-glucoside	6.20	287/352	447.0502	285.0361
Quercetin-3-*O*-rutinoside	6.23	237/353	609.1484	301.0297
Quercetin-3-*O*-galactoside	6.35	237/352	463.0870	301.0255
Quercetin-3-*O*-glucoside	6.51	237/350	463.0914	301.0255
Protocatechuic acid derivatives	7.14	269	425.0900	197/153/109
Keampferol-*O*-dihexoside	7.60	350	609.2394	285.0395
Procyanidin dimer	9.00	284	577.2555	289.0578
Quercetin-triglucoside	9.84	238/353	625.2783	301.1220

Table 2. Quantification of phenolic compounds [mg (100 g)$^{-1}$ dm] found in pistachios as affected by rootstock (*P. Integerrima* = INT, *P. Atlantica* = AT, and *P. Terebinthus* = TE) and irrigation treatment.

Compounds	ANOVA test[†]			Concentration [mg (100 g)$^{-1}$ dm]					
				Rootstock			Irrigation treatment		
	Rootstock	Irrigation	Root. x Irrig.	INT	AT	TE	T0	T1	T2
(+)-Catechin	NS	***	NS	114	118	127	143 a[‡]	119 b	97.64 c
(-)-Epicatechin	NS	NS	NS	42.61	25.51	30.98	30.49	38.10	30.51
Deoxydihexoside	NS	NS	NS	9.96	11.41	11.25	11.29	11.28	10.06
Caffeic acid hexoside	**	NS	NS	7.71 b	8.28 b	9.18 a	8.20	8.83	8.23
Protocatechuic acid derivatives	NS	NS	NS	7.76	8.67	8.13	8.37	8.75	7.43
Eriodictyol-3-*O*-glucoside	NS	NS	NS	2.40	2.23	2.10	2.19	2.27	2.27
Quercetin-*O*-galloyl-hexoside	NS	**	*	7.22	6.78	7.70	6.83 b	8.11 a	6.74 b
Keampferol-3-*O*-glucoside	NS	***	*	1.60	1.71	1.74	1.60 b	1.84 a	1.60 b
Quercetin-3-*O*-rutinoside	NS	NS	NS	3.97	4.30	4.40	3.92	4.69	4.06
Quercetin-3-*O*-galactoside	NS	NS	NS	4.24	4.22	4.37	4.12	4.34	4.37
Quercetin-3-*O*-glucoside	NS	NS	NS	4.70	5.45	4.98	5.05	5.14	4.94
Keampferol-*O*-dihexoside	***	NS	***	1.26 b	1.19 b	1.91 a	1.24	1.34	1.14
Quercetin-triglucoside	NS	NS	*	1.39	1.60	2.90	2.75	1.58	1.56
Cyanidin-3-*O*-galactoside	NS	NS	NS	16.35	15.51	14.59	14.67	15.92	15.87
Cyanidin-3-*O*-glucoside	NS	NS	NS	3.31	3.88	3.51	3.39	3.75	3.56
Polymeric procyanidins	***	***	***	323 b	415 a	443 a	352 b	427 a	404 a
Total	**	***	**	554 b	636 a	680 a	603 b	664 a	604 b

[†]NS = not significant at p<0.05; *, **, and ***, significant at p< 0.05, 0.01, and 0.001 respectively. [‡]Values (mean of 3 replications) followed by the same letter, within the same row and factor (rootstock or irrigation treatment), were not significantly different (p < 0.05), according to the Tukey's least significant difference test.

Table 3. Determination of triterpenoids (μg g^{-1} dm) found in pistachios as affected by rootstock and irrigation treatment.

Factors	Triterpenoids (μg g^{-1} dm)			
	Betulinic acid	**Oleanolic acid**	**Ursolic acid**	**Total**
		ANOVA test[†]		
Rootstock	*	NS	NS	NS
Irrigation	NS	NS	NS	NS
Root. x Irrigat.	NS	NS	NS	NS
		Tukey´s multiple range test[‡]		
Rootstock				
P. Integerrima	80.9 b	62.5	472	616
P. Atlantica	102 b	57.6	456	616
P. Terebinthus	111 a	60.5	528	700
Irrigation				
T0	108	63.6	551	723
T1	93.3	73.0	451	617
T2	92.9	44.1	455	592

[†]NS = not significant at p< 0.05; * significant at p< 0.05. [‡]Values (mean of 3 replications) followed by the same letter, within the same column and factor (rootstock or irrigation treatment), were not significantly different (p<0.05), according to Tukey's least significant difference test.

Table 4. Determination of α-amylase inhibition IC_{50} (mg dried nut mL^{-1}) in pistachios as affected by rootstock and irrigation treatment.

Factors	α-Amylase inhibition IC_{50} (mg dried nut mL^{-1})
	ANOVA test†
Rootstock	NS
Irrigation	*
Root. x Irrigat.	NS
	Tukey´s multiple range test‡
Rootstock	
P. Integerrima	24.00
P. Atlantica	23.90
P. Terebinthus	23.42
Irrigation	
T0	19.73 b
T1	26.42 a
T2	25.17 a

†NS = not significant at p< 0.05; * significant at p< 0.05. ‡Values (mean of 3 replications) followed by the same letter, within the same column and factor (rootstock or irrigation treatment), were not significantly different (p<0.05), according to Tukey's least significant difference test.

Capítulo 5

Estudio de las propiedades antimutagénicas y citoprotectivas

PUBLICATION 6

EFFECT OF ROOTSTOCK AND REGULATED DEFICIT IRRIGATION ON ANTIOXIDANT, ANTIMUTAGENIC, AND CYTOPROTECTIVE PROPERTIES OF PISTACHIOS

Luis Noguera-Artiaga, Joel Said García-Romo, Ema C. Rosas-Burgos, Francisco Javier Cinco-Moroyoqui, Reyna Luz Vidal-Quintanar, Ángel Antonio Carbonell-Barrachina, Armando Burgos-Hernández

Molecules, 2019

(En revisión)

Índice de calidad

Journal Citation Reports (2018)

Área: Biochemistry & Molecular Biology

Factor de impacto: 3.060 (136/299)

Cuartil: Q2

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Journal: Molecules
Manuscript ID: molecules-648317
Title: Antioxidant, antimutagenic, and cytoprotective properties of hydroSOS pistachio nuts
Authors: Luis Noguera-Artiaga , Joel Said García-Romo , Ema C. Rosas-Burgos , Francisco Javier Cinco-Moroyoqui , Reyna Luz Vidal-Quintanar , Ángel Antonio Carbonell-Barrachina , Armando Burgos-Hernández *

Received: 05 November 2019
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EFFECT OF ROOTSTOCK AND REGULATED DEFICIT IRRIGATION ON ANTIOXIDANT, ANTIMUTAGENIC, AND CYTOPROTECTIVE PROPERTIES OF PISTACHIOS

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Running title: Biofunctional properties of pistachios

ABSTRACT

Cancer is one of the leading causes of death in the world. Nowadays, a number of plant-based agents are clinically used in cancer treatment. Pistachio nuts are included among foods with the highest antioxidant capacity due to its high phenolic content. Stressed cultivating conditions such as the use of regulated deficit irrigation (RDI) is expected to create a plant response that might increase the production of secondary metabolites. The aim of this work was to study the influence of using different rootstocks (*P. atlantica*, *P. integerrima*, and *P. terebinthus*) and the application of 2 RDI treatments on the antioxidant (ABTS, FRAP, and DPPH), antimutagenic (Ames test), and cytoprotective (MTT assay in 5 human cell lines) activities of pistachios. *P. terebinthus* showed the best antioxidant activity, and RDI treatments maintained and improved the antioxidant properties of pistachios. Neither the rootstock nor the irrigation treatment had had significant impact on the antimutagenic potential of pistachios. Nut extracts had no toxic effect on non-cancerous cells and the application of RDI did not reduced their cytoprotective capacity. Furthermore, neither rootstock nor RDI treatments affected the ability of the pistachio extracts of preventing the oxidative damage by H_2O_2. The application of RDI strategies in pistachio orchards, in addition to allow irrigation water saving, led to obtain hydroSOS nuts with the same or even better biofunctional characteristics as compared to control pistachios coming from fully irrigated pistachio trees.

PRACTICAL APPLICATIONS: The application of regulated deficit irrigation in pistachios, allows farmers to save water during cultivation and produce pistachios with the same or better functional characteristics.

KEYWORDS: Ames test; cancer; hydroSOStainable products; MTT assay; *Pistacia vera*.

1. INTRODUCTION

The genus *Pistacia* belongs to the *Anacardiaceae*, a family that comprises about 70 genera and over 600 species. *Pistacia vera* is the only species of the genus commercially cultivated, and the rest of the species are mostly used as rootstocks (Bozorgi et al., 2013). As pistachio cultivation requires the use of rootstock, it is essential to study its influence on nut quality and functional activity. The main pistachio rootstocks are (i) *P. terebinthus* L. in the Mediterranean basin, (ii) *P. integerrima* L. and (iii) a hybrid between *P. integerrima* Steward ex Brandis and *P. atlantica* Desf. (UCB-I) in the USA, and *P. vera* L. in Iran (Gijón et al., 2010; Moriana et al., 2019).

Water is a scarce commodity and all productive sectors depend on it, especially agriculture. Therefore, it is necessary to improve the efficiency of its use. Regulated deficit irrigation (RDI) is a system of managing water supply by imposing different levels of water deficit at specific phenological stages, which have been found to be less sensitive, with no or low yield reduction and consequently low or no loss of economic benefits to farmers (Gijón et al., 2011). Vegetables and fruit cultivated under RDI are marketed under the brand "hydroSOStainable or hydroSOS" products. This food category is characterized by its environmental respect (optimized use of irrigation water) and by a theoretical increase of secondary metabolites, which will improve functionality and quality (Noguera-Artiaga et al., 2016). To be able to categorize a product as hydroSOS, it is necessary that it meets strict water control requirements during cultivation. This characterization is based on 16 hydric indicators (each one providing different marks or scores) and their sum allows classifying orchard management as a hydroSOS one or not (Corell et al., 2019).

The application of RDI depends on the phenological phases of each crop. In the case of pistachio, 3 different phases can be easily distinguished during fruit development: (i) the nut grows up to its maximum size, (ii) the shell hardens, and (iii) the growth of the edible part occurs. The second stage is the one at which the pistachio nut is less sensitive to water stress; thus, strategies aimed at reducing water supply during the cultivation of this nut must be applied at this specific time period .

Cancer is one of the leading causes of death in the world. It is caused by environmental factors such as carcinogens, viruses, chemicals, and radiation as well as by a genetic history (Namvar et al., 2012). Exposure to mutagens is one of the main causes of cancer development, causing the mutation of genes directly involved in the regulation of the cell cycle. The five types of cancer that cause the highest number of deaths are lung, colorectal, stomach, liver, and breast (World Health Organization, 2018). Many natural products have the potential to trigger apoptosis

in numerous human cancer cell types. Nowadays, a number of plant-based agents are clinically used in cancer treatment proving the goodness of these type of products. Hence, it is necessary to search for new plant-derived products as apoptosis inducers (Cragg & Newman, 2005; Khazir, Mir, Pilcher, & Riley, 2014).

Pistachio nuts have been recently ranked among the first 50 highest antioxidant food products and a rich source of phenolic compounds (Dreher, 2012). Pistachio contains epicatechin, quercetin, kaempferol, cyaniding-3-*O*-galactoside, cyanindin-3-*O*-glucoside, among other polyphenols (Noguera-Artiaga, Pérez-López, Burgos-Hernández, Wojdyło, & Carbonell-Barrachina, 2018). It has been shown that pistachio polyphenols are bioaccesible during simulated human digestion, releasing more than 90 % of its total content in the gastric compartment (Mandalari et al., 2013). Consequently, pistachios have a high number of bioactive compounds and these can be assimilated and used by the human body.

The main aims of this work were i) to study the antioxidant, antimutagenic, and cytoprotective properties of pistachios, and ii) the influence of the rootstock (n=3) and a decrease in the application of irrigation water during cultivation on their main functional activity.

2. MATERIAL AND METHODS

2.1. Plant material and experimental design

The experiment was conducted on the experimental farm "El Chaparrillo", Ciudad Real, Spain (L 3°56'W; L 39°0'N; altitude 640 m) during the crop season 2016-2017. The plant material consisted of pistachio trees *P. vera* L. cultivar Kerman was budded on three rootstocks (i) *P. terebinthus* L., (ii) *P. atlantica* Desf., and (iii) *P. integerrima* L. Tree-spacing was set at 7 × 6 m (238 trees ha^{-1}). Peter cv. was used as male tree and was evenly distributed throughout the field, in a proportion of 10 %. The climate of the area had an average annual rainfall of 397 mm, mostly distributed outside a 4-month summer drought period, and the surface is a shallow 1.3 m deep clay-loam (Alfisol Xeralf Petrocalcic Palexeralfs) soil, and a discontinuous petrocalcic horizon of around 0.75 m. The orchard was managed under no tillage conditions; weeds were controlled with post-emergence herbicides. Pest control and fertilization practices were those usually followed by local growers.

Control plants (T0) were irrigated at 100 % of crop irrigation requirements (ETc) of the previous week, according to daily reference evapotranspiration (ETo), a crop factor and taking into consideration the canopy size (Memmi, Gijón, Couceiro, & Pérez-López, 2016). In addition to T0, two RDI treatments (T1 and T2) were applied during stage II (fruit growth), the non-critical period. In these treatments, the water

deficit was increased and the threshold values were −1.5 MPa (T1) and −2.0 MPa (T2) (Gijón et al., 2011).

2.2. Extraction of functional compounds

The preparation of pistachio extracts was conducted as previously described by Noguera-Artiaga et al. (2018). One gram of grounded pistachio was weighed and combined with 5 mL of hexane and 8 mL of 30 % of aqueous methanol (v/v) amended with 1 % of ascorbic acid. The suspension was stirred, sonicated during 15 min in an ultrasonic bath (JP Selecta S.A, model 3000512 Barcelona, Spain) with constant frequency (40 kHz), left overnight at room temperature at darkness, and centrifuged during 10 min (20.878×*g* at 4 °C). The hydrophilic phase was separated, dried under N_2 stream, re-dissolved with sterile DMSO (solution of 100 mg mL^{-1}), and stored (-24 °C) until the analyses. This extraction methodology has showed to be one of the most effective ones when it comes to extracting the greatest number of phenolic compounds (Rajaei, Barzegar, Mobarez, Sahari, & Esfahani, 2010).

2.3. Antioxidant tests

Free radical scavenging capacities were determined by ABTS (Re et al., 1999), DPPH (Brand-Williams, Cuvelier, & Berset, 1995) and FRAP (Benzie & Strain, 1996) assays, with some modifications to be used in 96-well microplates.

The evaluation of antioxidant activity with ABTS reagent [2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was carried out according the method previously described by Loarca-Piña, Mendoza, Ramos-Gómez, and Reynoso (2010). Briefly, 20 µL of pistachio extracts were mixed with 230 µL of ABTS solution, and after reaction, absorbance was recorded at 734 nm in a Beckman Coulter AD 340 Microplate reader (Beckman Coulter, CA, USA).

In order to performed the DPPH assay, a 150 µM DPPH solution was prepared in 80 % methanol. A 200-µL aliquot of this solution was combined with 100 uL of pistachio extract, placed into each well and the plate was covered and left at darkness at room temperature for 6 min. After reaction, absorbance was determined at 520 nm in a Beckman Coulter AD 340 Microplate reader (Cardador-Martínez, Loarca-Piña, & Oomah, 2002).

The ferric reducing antioxidant power (FRAP) assay measures cability of reducing ferric ions. FRAP reagent was freshly prepared by mixing 100 mL of a sodium acetate buffer (0.3 M, pH 3.6) solution, 10 mL of 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution (10 mM in 40 mM HCl), and 10 mL of $FeCl_3$ $6H_2O$ (20 mM) solution. A 200-µL aliquot of the pistachio extract was mixed with FRAP solution for 30 min at darkness in order to obtain a proper and even reaction. Absorbance of samples was

recorded at 593 nm using a Beckman Coulter AD 340 Microplate reader (Bolanos de la Torre, Henderson, Nigam, & Owusu-Apenten, 2015). Trolox was used as a standard antioxidant. All the analyses were run in triplicate and results were expressed as mM Trolox equivalents g^{-1}.

2.4. Antimutagenicity test

The antimutagenic activity of pistachio extract was evaluated using the standard mutagenicity assay described by Maron and Ames (1983) with *Salmonella typhimurium* TA100 as a tester strains, in the presence of sodium azide (SA) as positive control. Pistachio extracts were diluted in sterile DMSO and spiked with sufficient SA to reach 20.0, 2.0, and 0.2 μg mL^{-1}. All assays were performed in triplicate. Antimutagenic activity was reported as the percentage of SA-induced revertants per plate inhibited due to the presence of pistachio extract.

2.5. Cell lines

Human cell lines A-549 (lung carcinoma), HeLa (epithelial cervix adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), HCT 116 (colon carcinoma), and ARPE-19 (retinal non-cancerous epithelial) were used. Cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in Dulbecco's modified Eagle's medium and RPMI-1640 Medium (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10 and 15 % heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and grown at 37 °C in an atmosphere of 5 % CO_2.

2.6. Cytotoxic test

To study the cytotoxic effect of pistachio extracts on human cancer cell lines MTT assay (Roche, cell proliferation kit I, Roche, Cat. No. 11-465-007-001) was used. In a 96-flat-well plate $1x10^4$ cells $well^{-1}$ were seeded and suspended in 100 μL of medium. After 24 h of incubation at 37 °C and 5 % CO_2 atmosphere, cell cultures were maintained for another 24 h at same conditions with the addition of 100 μL of culture medium without FBS and the pistachio extracts re-suspended in DMSO (0.5 %) at final a concentration of 100 μg mL^{-1}. Control cell cultures did not show any evidence of cell damage. Cisplatin [*cis*-Diamminedichloroplatinum(II)] was used as a positive control for cytotoxicity at a concentration of 50 μg mL^{-1} for all the cell lines studied, except for MDA-MB-231 in which case the concentration was 100 μg mL^{-1}. The plates were read the next day using an ELISA plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) (Fathalizadeh et al., 2015).

2.7. Cytoprotection against H2O2-induced cell damage activity

In order to find the H_2O_2 concentration to be used to caused oxidative cell damage, a dose response curve to determine cellular viability in the presence of H_2O_2 was prepared. ARPE-19 cells were seeded ($1x10^4$ cells $well^{-1}$) into 96-well plates, suspended in 100 µL of DMEM and incubated for 24 h at 37 °C and 5 % CO_2 atmosphere. Then, cells were treated with different concentrations of H_2O_2 (0.062, 0.125, 0.250, 0.5, 1.0, 10.0, and 20.0 mM) for 30 min. After the treatment, the cell medium was replaced (by an equal volume of DMEM serum-free medium) and the original MTT protocol was followed.

When the concentration of H_2O_2 to be used was known, the analysis of the protection against reactive oxygen species was conducted. ARPE-19 cells, $1x10^4$ cells $well^{-1}$, were plated in 96-well plates for 24 h (suspended in 100 µL of DMEM, at temperature of 37 °C and controlled atmosphere with 5 % CO_2). Then, cells were incubated with extracts obtained from pistachios cultivated under different irrigation treatments and using different rootstocks, at a concentration of 100 µg mL^{-1}, during 4 and 24 h. After that, medium was retrieved from wells and they were incubated with 10 mM of H_2O_2 for 30 min (at same temperature and atmosphere). After treatment, the cell medium was replaced by equal volume of DMEM serum-free medium and MTT assay was performed (Hu, Liang, Zhao, & Wang, 2019; Liu et al., 2017).

2.8. Statistical analysis

The data presented in this study are the mean values of, at least, 3 replicates and was subjected to three-way (in case of antimutagenicity test) and two-way (rest of determinations) analysis of variance (ANOVA). Then, data were subjected to Tukey's multiple-range test to compare the means. Differences were considered statistically significant at $p<0.05$. All statistical analyses were done using XLSTAT software (version 2014.1).

3. RESULTS AND DISCUSSION

3.1. Antioxidant tests

The $ABTS^+$ method, based on the capability of presumptive antioxidant to reduce the $ABTS^{+\bullet}$ radical, is one of the most widely used methods because uses a stable reaction, is practical, highly sensitive, and fast to carried out. Additionally, this method allows confirmation of antiradical capacity of either hydrophilic and lipophilic antioxidants (Rakholiya, Kaneria, & Chanda, 2017). The free radical scavenging capacities of $ABTS^{+\bullet}$ showed that pistachios obtained using the *P. terebinthus*

rootstock had the highest antioxidant activity (3.67 mM TE g^{-1}), followed by *P. integerrima* (3.23 mM TE g^{-1}), and finally *P. atlantica* (2.80 mM TE g^{-1}) (**Table 1**). In the case of pistachios obtained under different irrigation treatments, nuts from T2-trees had the higher antioxidant activity than samples from trees subjected to the other two irrigation treatments (T0 and T1), with an antioxidant activity of 3.92 mM TE g^{-1} *versus* 2.86 and 2.96 mM TE g^{-1}, respectively (**Table 1**). In a study evaluating key characteristics of 8 pistachio cultivars by Noguera-Artiaga et al. (2019), similar values of antioxidant activity for the Kerman cultivar were found, the same cultivar used in the current experiment.

The antioxidant activity of the hydrophilic fraction of pistachios was measured using the FRAP method, which is based on the ability of an antioxidant to reduce Fe^{+3} in the presence of 2,4,6-Tripyridyl-s-triazine (TPTZ), forming a Fe^{+2}-TPTZ complex (Kaneria, 2017). After the analysis of samples obtained using different rootstocks (**Table 1**), samples *P. integerrima* and P. t*erebinthus* had the highest antioxidant activity with values of 1.37 and 1.32 mM TE g^{-1}, respectively; while nuts from *P. atlantica* had 1.23 mM TE g^{-1}. Taking into account the irrigation treatment factor, the T1-sample had the highest antioxidant activity with 1.37 mM TE g^{-1}, followed by a group consisting of the samples T0 and T2 (1.29 mM TE g^{-1}). Similar results were obtained by Taghizadeh et al. (2018) after analyzing the antioxidant activity by FRAP method of pistachio extracted using different solvents.

As shown in **Table 1**, statistically differences were also found after the analysis of the antioxidant activity of pistachio extracts using the DPPH$^{\bullet}$ method. Pistachios obtained on *P. terebinthus* rootstock showed the highest antioxidant activity (5.63 mM TE g^{-1}), while the *P. integerrima* samples had the lowest value (5.07 mM TE g^{-1}). Regarding the irrigation treatments, the DPPH method was the most effective one to differentiate among samples. The antioxidant activity increased with water restriction (deficit irrigation); control sample (T0) showed the lowest antioxidant activity (4.59 mM TE g^{-1}), followed by T1 samples (5.07 mM TE g^{-1}) and finally T2 samples had the highest value (6.41 mM TE g^{-1}).

In view of results above described, it can be concluded that the use of the *P. terebinthus* rootstock led to the highest antioxidant activity (according to the 3 methods used). This can be associated to the fact that *P. terebinthus* rootstock is, among the 3 rootstocks studied, the one with the highest polyphenolic content and highest amount of betulinic acid, according to Noguera-Artiaga et al. (2018). Besides, it can be concluded as well that, deficit irrigation treatments (T1 and especially T2) maintained or improved the antioxidant (hydrophilic) capacity of pistachios. In plants cultivated at water deficit conditions, an excess of CO_2 assimilated could have

increased the biosynthesis of carbon-based secondary metabolites when carbohydrates exceed the amount used for growth concentrations (Horner, 1990).

3.2. Antimutagenicity test

The number of histidine^{+} revertant colonies obtained were 120 ± 5 (spontaneous revertants) while, in the presence of the mutagen (sodium azide, 30 µg plate^{-1}), 1652 ± 33 revertants plate^{-1} were counted. Samples inhibited an average of 8 % of the mutagenicity (92% revertants) induced by SA, without significant differences with control (**Figure 1**). No differences were observed for irrigation treatment and rootstock. The pistachio extracts were neither toxic nor mutagenic to the bacteria at tested concentrations, and bacterial growth was normal. Similar results were obtained by Rajaei et al. (2010) when the antimutagenic activity of pistachio green hull extract was assayed, since they found that hydrophilic pistachio extracts (raw, unpurified) did not show antimutagenic activity, although in this case the study was carried out using 2-nitrofluorene as a mutagen control instead of sodium azide.

These results showed that phenolic compounds of pistachio extracts had no activity against sodium azide. The application of different irrigation treatments or different rootstocks had no significant impact on the antimutagenic potential of pistachios.

3.3. Cytotoxic test

In vitro cytotoxicity assessment is increasingly being recognized as an effective indicator of the toxic potential of compounds against cancerous cells. The MTT test assesses cellular viability measuring intracellular reduction of MTT reagent (water soluble and yellow) to a formazan salt (water insoluble and purple), which can be colorimetrically detected (Pacifico et al., 2012).

The effect that regulated deficit irrigation and rootstocks may have had on the functional properties of pistachios was studied by evaluating the cytotoxic effect of pistachio extracts on 4 cancerous and 1 non-cancerous cell lines (**Table 2**). Results obtained from testing samples were compared to those obtained with cisplatin, a positive cytotoxic control agent, which is a genotoxic drug used in chemotherapy. In two of the cancerous cell lines under study (HCT-116 and MDA-MB-231) no statistically differences were found associated to neither rootstock nor irrigation treatment factors. In the case of the HCT-116 cell line, the pistachio samples analyzed caused an inhibition of ~ 60 % (~ 40 % viability), achieving the same effect as a cisplatin dose of 50 µg mL^{-1} did. The same effect was observed in the MDA-MB-231 cell line with a cellular viability of ~ 60 % (**Table 2**).

In A549 and HeLa cell lines, no statistically differences were observed among samples from different irrigation treatments (**Table 2**). However, rootstock had significant differences as compared to the control (cisplatin, 50 mg mL^{-1}), with pistachio samples having statistically higher viability (average ~ 68 and ~ 61 % of viability in A549 and HeLa lines, against ~ 61 and ~ 57 %, respectively) than when cisplatin was used.

In ARPE-19 non-cancerous cell line, no differences were found among samples obtained under different irrigation treatments, but significant differences were found when the rootstock factor was analyzed (**Table 2**); the *P. atlantica* rootstock led to the highest cellular viability (94 %). In addition, the 3-rootstock samples had higher cellular viability than that observed for the control treatment. An important issue to point out is that none of the pistachio extracts reduced cellular viability of ARPE-19 below 85 %; thus, they cannot be considered cytotoxic up to 100 µg mL^{-1}.

These results showed that the compounds present in pistachio extracts had no toxic effect on ARPE-19 non-cancerous cells. On the contrary, they could significantly affect all 4 human cancer lines, with A549 cell line being the most resistant and HeLa the most sensitive. Similar results have been previously reported Seifaddinipour, Farghadani, Namvar, Mohamad, and Abdul Kadir (2018) on extracts from pistachio hulls on 6 cancerous cell lines and normal fibroblast, in which the same methodology used in the present study was followed. On the other hand, the application of regulated deficit irrigation treatments had not diminished the original cytotoxic capacity that control pistachios have on the carcinoma cell lines studied.

It has been shown in other studies (Noguera-Artiaga et al., 2018) that neither regulated deficit irrigation nor the use of different rootstocks did affect the total concentration of triterpenoids. Pistachios are characterized by having triterpenoids; especially betulinic, oleanolic, and ursolic acids. Several studies have established relationships between phenolic compounds and their activity against tumor cell lines as tested *in vitro* (Novotný, Vachálková, & Biggs, 2001; Pięt & Paduch, 2019).

3.4. Cellular viability and oxidative damage tests

The lowest H_2O_2 concentrations (0.062 and 0.125 mM) tested had no significant effect on ARPE-19 cells as compared to the control (0 mM of H_2O_2), obtaining values close to 100 % cellular viability (**Figure 2**). Then, a significant negative relationship between the H_2O_2 concentration and the cellular viability was observed (R^2 = 0.85), with the viability reaching values of ~10 %. Based on these results, the concentration of 10 mM of H_2O_2 was chosen to perform the cellular damage test. This selection was based on the fact that this concentration led to a cellular viability close to 20 %,

causing obvious damage to the cells but allowing enough cell survival to guarantee that test would be perform at optimal conditions for its full development.

The results obtained after incubation of ARPE-19 cells during 4 and 24 h with the pistachio extracts are shown in **Figure 3**. As previously discussed, when 10 mM of H_2O_2 was used, cellular viabilities of ~ 20 % were obtained, without having significant differences among the studied samples. When the cells were incubated with the pistachio extracts, no statistically differences were found for the irrigation treatments, leading to ~ 95 % cellular viability. However, the *P .atlantica* rootstock led to a higher cellular viability (~ 100 %) as ~ 95 % for *P. integerrima* and *P. terebinthus*. When H_2O_2 (10 mM) was added to the cells for 30 min (after 4 h of incubation with the pistachio extracts), a viability of ~ 30 % was obtained in all samples. This suggest that the compounds present in pistachio extracts were able to reduce the oxidative damage observed in ARPE-19 cells by approximately 10 % after 4 h (**Figure 3a**).

By repeating the same process but incubating ARPE-19 cells with the pistachio extracts for a longer time, 24 h, results were similar to those obtained after 4 h of incubation. There were no significant differences among irrigation treatments but *P. atlantica* showed a better behavior (~ 95 % cellular viability) as compared to ~ 85 % for *P. integerrima* and *P. terebinthus* (**Figure 3b**). When H_2O_2 (10 mM) was added to the cells for 30 min (after 24 h of incubation with the pistachio extracts), a viability of ~ 55 % was obtained in all samples (without statistically differences among them). In this case, pistachio extracts were able to protect ARPE-19 cells from oxidative damage by 35 %, approximately. The cellular internalization depends on exposure time of cell to pistachio extracts. Increasing the exposure time, increases the amount of pistachio compounds into the cell, so that greater protection against oxidative stress caused by peroxide radicals (formed from H_2O_2 by Fenton or Haber-Weiss reactions) was achieved.

Based on the above, neither rootstock nor regulated deficit irrigations significantly affected the original protective level against oxidative damage by H_2O_2 of the control pistachio extracts.

4. CONCLUSIONS

Experimental results have demonstrated that use of the *P. terebinthus* rootstock led to pistachios with higher antioxidant activity, while application of RDI strategies maintained or improved pistachios antioxidant capacity. Neither the use of different rootstocks nor application of RDI during pistachio cultivation had significant impact on the antimutagenic potential and cytoprotective activity of pistachios. Of

the 4 cancer cell lines studied, A549 was the most resistant and HeLa cell line was the most sensitive to pistachio extracts, which successfully protected ARPE-19 cells from oxidative damage caused by H_2O_2 at a level of 10 % when incubated during 4 h, and at 35 % after 24 h of exposure. Thus, the application of RDI strategies in pistachios allows the saving of water during pistachio cultivation and led to produce pistachio nuts with the same or better functional characteristics than control samples.

ACKNOWLEDGMENTS

Luis Noguera-Artiaga was funded by an FPU grant from the Spanish government (FPU014/01874)

AUTHORS' CONTRIBUTIONS

L. Noguera-Artiaga and J. S. García-Romo contributed to experimental design, collected data, interpreted the results and drafted the manuscript. E. C. Rosas-Burgos, F. J. Cinco-Moroyoqui, and R. L. Vidal-Quintanar contributed to experimental design and result interpretation. Á. Á. Carbonell-Barrachina and A. Burgos-Hernández interpreted the all results and revised the manuscript. All authors discussed and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1-Antioxidant activity [mM Trolox equivalents (TE) g^{-1}] of pistachio extracts as affected by rootstock and irrigation treatment.

Factor	ABTS	FRAP	DPPH
	(mM TE g^{-1})		
	ANOVA[1]		
Rootstock	***	***	***
Irrigation	***	**	***
Rootstock x Irrigation	***	**	**
	Tukey multiple range test[2]		
Rootstock			
Atlantica	2.80 c	1.23 b	5.44 ab
Integerrima	3.23 b	1.37 a	5.07 b
Terebinthus	3.67 a	1.32 a	5.63 a
Irrigation			
T0	2.86 b	1.29 b	4.59 c
T1	2.96 b	1.37 a	5.13 b
T2	3.92 a	1.29 b	6.41 a
Rootstock x Irrigation			
Atlantica x T0	2.37 d	1.24 ab	4.83 d
Atlantica x T1	2.61 d	1.34 a	6.07 b
Atlantica x T2	3.41 b	1.10 b	5.42 c
Integerrima x T0	2.95 cd	1.36 a	4.43 e
Integerrima x T1	2.90 cd	1.41 a	4.42 e
Integerrima x T2	3.94 ab	1.37 a	6.36 b
Terebinthus x T0	3.24 bc	1.28 a	4.51 e
Terebinthus x T1	3.36 bc	1.39 a	4.89 d
Terebinthus x T2	4.40 a	1.26 a	7.47 a
Pooled variance	0.32	0.15	0.22

[1]NS: not significant at $p < 0.05$; **, and ***, significant at $p < 0.01$, and 0.001, respectively.
[2]Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test.

Table 2-Viability (%) of pistachio extracts as affected by rootstock and irrigation treatment, at 100 µg mL^{-1} on human cancerous and non-cancerous cell lines.

Factor	HCT-116	A549	HeLa	MDA-MB-231	ARPE-19
	(% viability)				
	ANOVA[1]				
Rootstock	NS	***	**	NS	***
Irrigation	NS	NS	NS	NS	***
Rootstock x Irrigation	NS	***	***	NS	***
	Tukey multiple range test[2]				
Rootstock					
Atlantica	38.9	67.4 a	30.1 a	61.3	94.2 a
Integerrima	39.0	68.3 a	30.1 a	61.9	87.9 b
Terebinthus	43.4	68.7 a	32.3 a	60.3	86.6 b
CISP[§]	40.2	61.1 b	28.2 b	57.2	69.2 c
Irrigation					
T0	39.7	65.3	28.3	58.0	91.7 a
T1	39.8	64.4	27.0	60.8	87.7 a
T2	40.9	61.1	28.2	61.0	89.3 a
CISP[§]	40.2	61.1	28.2	57.2	69.2 b
Rootstock x Irrigation					
Atlantica x T0	36.1	73.2 a	30.1 a	62.5	99.1 a
Atlantica x T1	35.8	66.2 a	29.3 a	60.9	91.1 a
Atlantica x T2	44.3	64.6 ab	30.4 a	59.6	90.9 a
Integerrima x T0	36.3	74.5 a	28.4 ab	55.5	84.9 ab
Integerrima x T1	41.0	67.2 a	31.5 a	64.6	88.3 ab
Integerrima x T2	38.8	65.1 ab	29.6 a	64.9	90.6 a
Terebinthus x T0	46.7	63.7 ab	34.8 a	58.9	90.1 a
Terebinthus x T1	42.7	75.3 a	28.8 ab	59.8	83.8 ab
Terebinthus x T2	39.9	69.1 a	33.3 a	61.3	86.2 ab
CISP[3]	40.2	61.1 b	28.2 b	57.2	69.2 b
Pooled variance	4.1	2.3	1.8	4.8	4.5

[1]NS: not significant at $p < 0.05$; **, and ***, significant at $p < 0.01$, and 0.001, respectively. [2]Values (mean of 3 replications) followed by the same letter, within the same column and factor, were not significantly different ($p<0.05$), Tukey's least significant difference test. [3]Cisplatin (CISP) concentration was 50 µg mL^{-1} for all the cell lines studied, except for MDA-MB-231 in which case the concentration was 100 µg mL^{-1}.

Figure 1-Antimutagenicity test of pistachio extracts (% inhibition of sodium azide (SA) mutation) at different concentrations (0.2, 2.0, and 20.0 mg mL^{-1}). Values with same letters within a same factor were not significantly different (p<0.005). Spontaneous revertants 120 ± 5 and SA control (30 µg $plate^{-1}$) induced 1652 ± 33 revertants $plate^{-1}$.

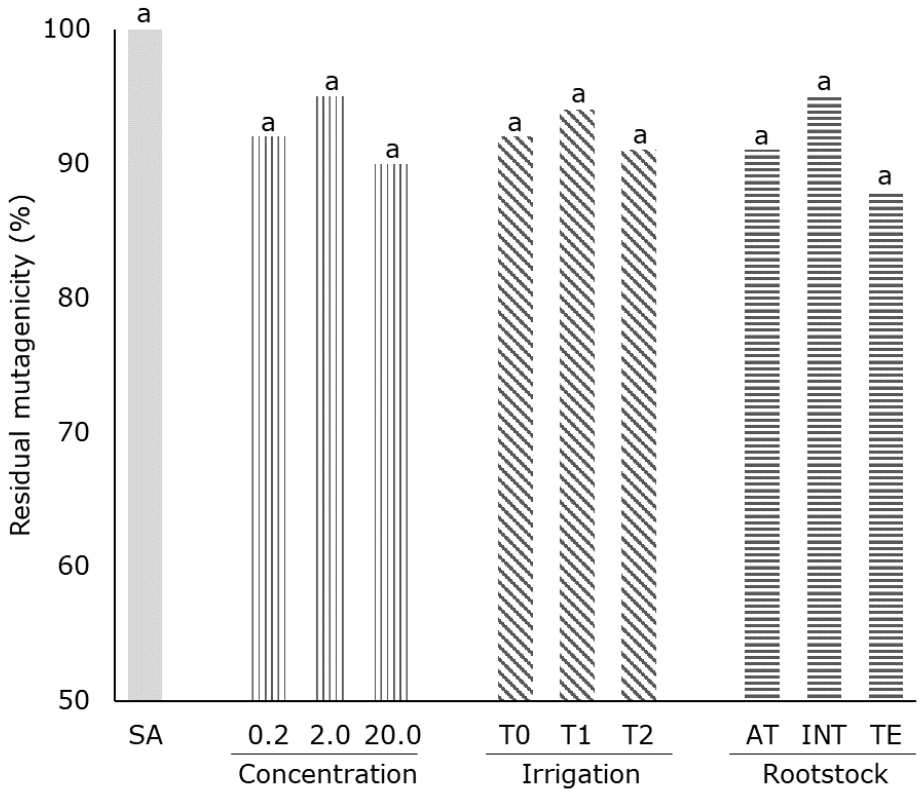


Figure 2-Cellular viability evaluated by MTT method in ARPE-19 cells exposed to different concentration of H_2O_2 during 30 min.

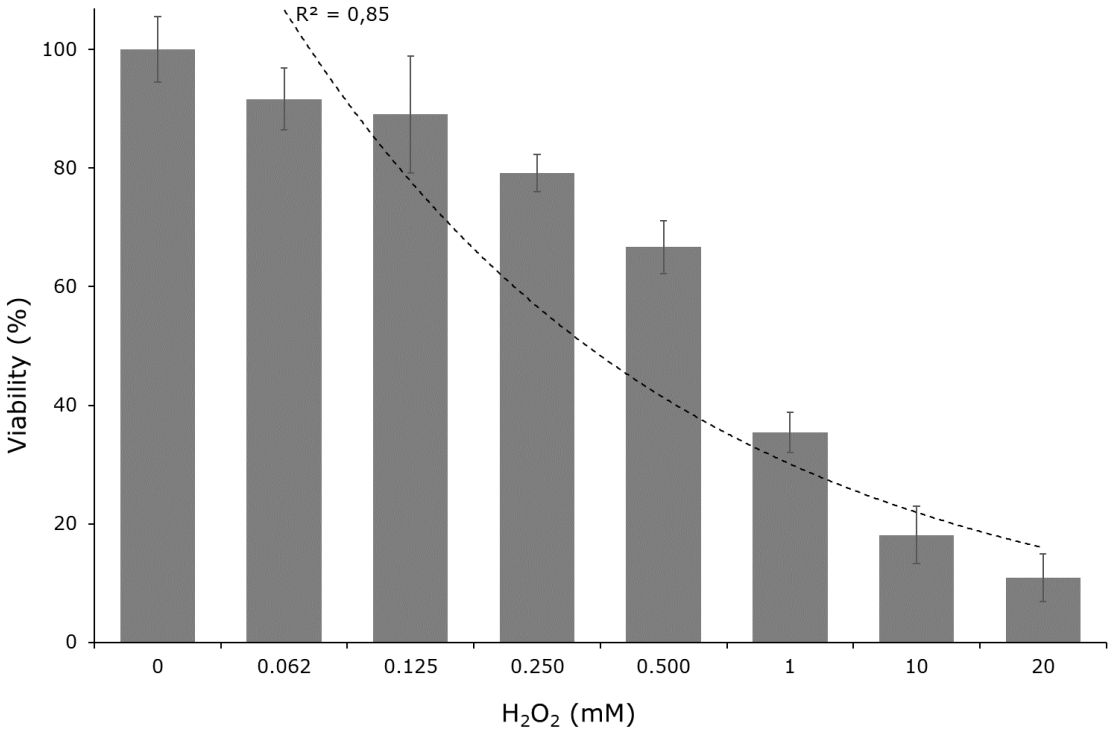
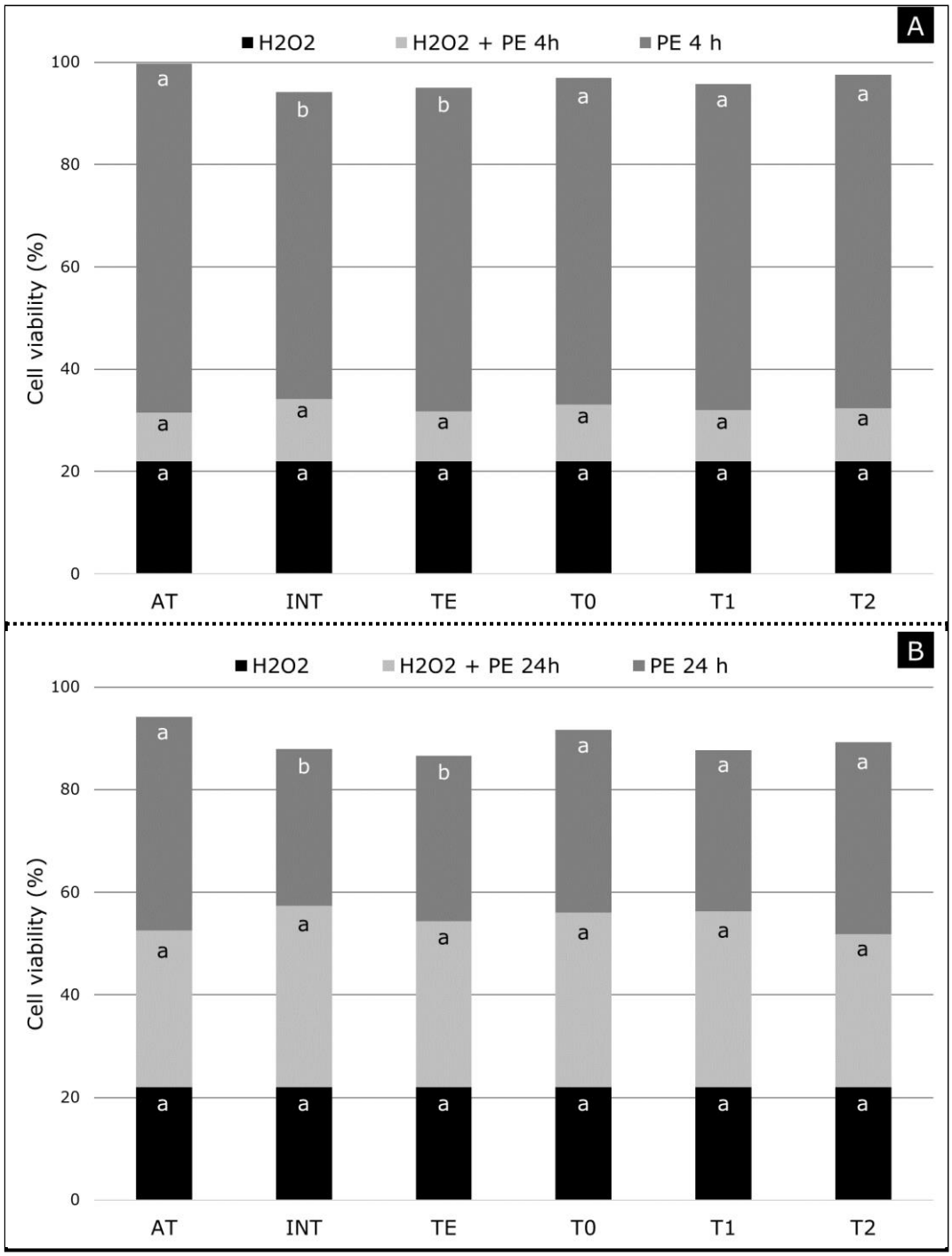


Figure 3-Cellular viability evaluated by MTT method of ARPE-19 cells treated with pistachio extracts (PE), as affected by rootstock and irrigation treatment, at 100 µg mL^{-1} after 4 h (Fig. 3A), and 24 h (Fig. 3B), and then exposed to 10 mM H_2O_2 during 30 min. Different letters within each factor means significant differences ($p \leq 0.05$); Tukey's least significant difference test.



CONCLUSIONES

De entre los 8 cultivares de pistacho estudiados, Kerman destacó por su funcionalidad y sus propiedades sensoriales, especialmente por una mayor intensidad de sabor a pistacho.

Los consumidores de frutos secos valoran positivamente los productos saludables, con buenas propiedades sensoriales y aquellos obtenidos respetando el medio ambiente, estando dispuestos a pagar hasta un 10 % más por un producto que cumpla dichas condiciones.

La aplicación de un tratamiento de riego deficitario moderado (T1, durante la fase fenológica II del cultivo del pistacho, el potencial hídrico del tallo se mantuvo en -1.5 MPa), produce pistachos con igual morfología, mejores atributos funcionales (entre los que destacan un mayor contenido de polifenoles), mejor calidad físico-química (gracias principalmente a un mejor perfil de compuestos volátiles) y mejor aceptación sensorial que los pistachos no estresados hídricamente; todo ello con un ahorro de más de un 30 % de agua y, en consecuencia, con una menor repercusión medioambiental y mayores beneficios económicos.

La aplicación de un tratamiento severo de riego deficitario controlado (T2, durante la fase fenológica II del cultivo del pistacho, el potencial hídrico del tallo se mantuvo en -2.0 MPa) tiene incidencia negativa en algunas propiedades claves en la calidad del pistacho, como el tamaño, la actividad antioxidante y las propiedades sensoriales.

La aplicación de tratamientos de riego deficitario controlado no merma las propiedades antimutagénicas y citotóxicas que los compuestos presentes en el pistacho tienen frente a células cancerígenas. Por otra parte, el riego deficitario controlado mantiene la capacidad de inhibición de α-amilasa característica de este fruto.

Entre los portainjertos estudiados, *P. terebinthus* es el que produce frutos de mayor calidad: frutos más grandes, de mayor peso, mejor textura, con mejor perfil de ácidos grasos, mejor capacidad antioxidante y mayor contenido total de polifenoles.

Con base en los resultados aquí descritos, la combinación surgida del empleo del portainjerto *P. terebinthus* y la aplicación de un riego deficitario controlado moderado (T1), aplicados al cultivar Kerman, es la mejor opción para obtener frutos con una mejor funcionalidad y calidad sensorial.

RECOMENDACIONES

Aplicar tratamientos de riego deficitario controlado manteniendo los niveles de estrés hídrico por debajo de -1.5 MPa durante un tiempo más prolongado, para estudiar si es posible reducir aún más, si cabe, el aporte hídrico manteniendo la calidad de los frutos obtenidos.

Fraccionar los extractos de pistacho con el fin de aislar los compuestos presentes y poder caracterizar más certeramente los compuestos responsables de sus propiedades citotóxicas y antioxidantes.

Implementar tratamientos de riego deficitario controlado en otros cultivos leñosos, con el fin reducir el aporte hídrico destinado a la agricultura y, al mismo tiempo, mejorar la calidad de los frutos obtenidos.