

UNIVERSITAT POLITÈCNICA DE VALÈNCIA
DEPARTMENT OF FOOD TECHNOLOGY



**HARVEST AND POSTHARVEST QUALITY OF
PERSIMMON FRUIT: PHYSICOCHEMICAL
AND NUTRITIONAL ASPECTS**

PhD Thesis

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*Sometimes you're ahead, sometimes you're behind.
The race is long and in the end, it's only with
yourself*

*To my parents who have worked hard allowing
me to achieve my goals and showing me that every
effort has its reward*

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RESUMEN

El caqui se ha convertido en los últimos años en un cultivo de gran relevancia en el área mediterránea de España, estando la producción centrada en una única variedad, el cv. Rojo Brillante, y localizada principalmente en la Comunidad Valenciana. Las principales alteraciones presentadas por el caqui ‘Rojo Brillante’ durante el periodo postcosecha son el pardeamiento de la pulpa asociado a los daños mecánicos y los daños por frío manifestados tras la conservación a bajas temperaturas. Las investigaciones previas han determinado las condiciones de manejo bajo las cuales se desarrollan dichas alteraciones, sin embargo los procesos bioquímicos involucrados en la manifestación de estos desórdenes no se conocen en profundidad.

Por otra parte, actualmente uno de los principales retos es la introducción de nuevas variedades que permitan ampliar la gama varietal, así como prolongar los periodos de conservación del caqui con el fin de poder escalonar la puesta en el mercado en función de la demanda.

En este contexto, en la presente Tesis se han abordado tres objetivos principales: 1) Estudiar los procesos bioquímicos implicados en los principales desórdenes postcosecha del caqui, prestando especial atención a los cambios en el sistema redox del fruto; 2) Evaluar diferentes tratamientos postcosecha para preservar la calidad del fruto durante la conservación frigorífica; 3) Evaluar la calidad físico-química y nutricional de diferentes variedades de caqui introducidas desde otros países para ampliar la gama varietal.

Estudios bioquímicos, cromatográficos y microestructurales, han revelado que el pardeamiento de la pulpa o “browning”, manifestado por la fruta que ha sufrido daños mecánicos tras la eliminación de la astringencia está asociado a un proceso de oxidación de taninos motivado por una situación de estrés oxidativo. Además se ha descrito una nueva alteración de la pulpa, “pinkish bruising”, manifestada por los frutos sometidos a daño mecánico con alto nivel de astringencia. También se ha evaluado la sensibilidad al pardeamiento de diferentes variedades introducidas desde otros países.

Además, se ha determinado la implicación del sistema redox del fruto en la manifestación de daños por frío en ‘Rojo Brillante’, y se han descrito los cambios en dicho sistema asociados al alivio de los daños mediante el tratamiento con 1-MCP.

Con el objetivo de prolongar la conservación del fruto se ha ensayado el uso de la atmósfera controlada basada en 4-5% O₂ + N₂ sobre las variedades 'Rojo Brillante' y 'Triumph', observándose que la respuesta a esta tecnología depende de manera muy importante de la variedad. Los resultados fueron especialmente positivos en el caso del cultivar 'Triumph', en el que la atmósfera ensayada permitió prolongar la conservación frigorífica hasta tres meses.

Otra tecnología ensayada para el control de los daños por frío fue la aplicación de choques con altas concentraciones de CO₂ en el cultivar no astringente 'Fuyu'. Este tratamiento alivió el principal síntoma de daño por frío manifestado por esta variedad, la gelificación de la pulpa. Este efecto se ha relacionado con la preservación de la estructura celular de la pulpa del fruto.

Estudios recientes han mostrado que el tratamiento con etil formato es altamente efectivo en el control de insectos durante la conservación y comercialización del caqui 'Fuyu', sin embargo este tratamiento provoca un ablandamiento del fruto mermando su calidad. En esta Tesis se ha demostrado que las aplicaciones de etil formato activan los genes de síntesis de etileno, siendo esta hormona mediadora del ablandamiento. Los estudios también revelaron que la aplicación de un tratamiento previo con 1-MCP reduce el ablandamiento del fruto asociado al etil formato, por lo que el uso combinado de ambos tratamientos puede ser considerado una alternativa para la desinfección de los frutos sin detrimento de su calidad.

Por último, el estudio de diez variedades introducidas desde otros países permitió seleccionar en base a su momento de maduración y respuesta al tratamiento de desastringencia aquellas variedades de mayor interés para ampliar la gama varietal. Además, se identificaron los principales compuestos nutricionales del caqui y se evaluó el efecto del tratamiento de desastringencia con CO₂ sobre los mismos.

RESUM

El caqui s'ha convertit en els últims anys en un cultiu de gran rellevància en l'àrea mediterrània d'Espanya, estant la producció centrada en una única varietat, el cv. Rojo Brillante, i localitzada principalment a la Comunitat Valenciana. Les principals alteracions presentades pel caqui 'Rojo Brillante' durant el període postcollita són l'enfosquiment de la polpa associat als danys mecànics i els danys per fred manifestats després de la conservació a baixes temperatures. Les investigacions prèvies han determinat les condicions de maneig sota les quals es desenvolupen estes alteracions, però els processos bioquímics involucrats en la manifestació d'estos desordres no es coneixen en profunditat.

D'altra banda, actualment un dels principals reptes és la introducció de noves varietats que permetin ampliar la gamma varietal, així com prolongar els períodes de conservació del caqui per tal de poder escalonar la posada en el mercat en funció de la demanda.

En este context, en la present Tesi s'han abordat tres objectius principals: 1) Estudiar els processos bioquímics implicats en els principals desordres postcollita del caqui, posant especial atenció als canvis en el sistema redox del fruit; 2) Avaluar diferents tractaments postcollita per preservar la qualitat del fruit durant la conservació frigorífica; 3) Avaluar la qualitat fisicoquímica i nutricional de diferents varietats de caqui introduïdes des d'altres països per ampliar la gamma varietal.

Estudis bioquímics, cromatogràfics i microestructurals, han revelat que l'enfosquiment de la polpa o "Browning", manifestat per fruita que ha patit danys mecànics després de l'eliminació de l'astringència està associat a un procés d'oxidació de tanins motivat per una situació d'estrès oxidatiu. A més s'ha descrit una nova alteració de la polpa, "pinkish bruising", manifestada pels fruits sotmesos a dany mecànic amb alt nivell d'astringència. També s'ha avaluat la sensibilitat a l'enfosquiment de diferents varietats introduïdes des d'altres països.

A més, s'ha determinat la implicació del sistema redox del fruit en la manifestació de danys per fred en 'Rojo Brillante', i s'han descrit els canvis en el sistema associats a l'alleujament dels danys mitjançant el tractament amb 1-MCP.

Amb l'objectiu de perllongar la conservació del fruit s'ha assajat l'ús de l'atmosfera controlada basada en 4-5% O₂ + N₂ sobre les varietats 'Rojo Brillante' i 'Triumph', observant-se que la resposta a esta tecnologia depèn de manera molt important de la varietat. Els resultats van ser especialment positius en el cas del cultivar 'Triumph', en què l'atmosfera assajada va permetre prolongar la conservació frigorífica fins a tres mesos.

Una altra tecnologia assajada per al control dels danys per fred va ser l'aplicació de xocs amb altes concentracions de CO₂ en el cultivar no astringent 'Fuyu'. Este tractament va alleujar el principal símptoma de dany per fred manifestat per esta varietat, la gelificació de la polpa. Este efecte s'ha relacionat amb la preservació de l'estructura cel·lular de la polpa del fruit.

Estudis recents han mostrat que el tractament amb etil format és altament efectiu en el control d'insectes durant la conservació i comercialització del caqui 'Fuyu', però este tractament provoca un estovament del fruit minvant la seua qualitat. En esta Tesi s'ha demostrat que les aplicacions d'etil format activen els gens de síntesi d'etilè, sent esta hormona medidora de l'estovament. Els estudis també van revelar que l'aplicació d'un tractament previ amb 1-MCP redueix l'estovament del fruit associat a l'etil format, per la qual cosa l'ús combinat de tots dos tractaments pot ser considerat una alternativa per a la desinfecció dels fruits sense detriment de la seua qualitat.

Finalment, l'estudi de deu varietats introduïdes des d'altres països va permetre seleccionar en base al seu moment de maduració i resposta al tractament de desastringència aquelles varietats de més interès per ampliar la gamma varietal. A més, es van identificar els principals compostos nutricionals del caqui i es va avaluar l'efecte del tractament de desastringència amb CO₂ sobre els mateixos.

ABSTRACT

In recent years, persimmon crop has become very relevant in Mediterranean Spain, where the production of this fruit centres on only one variety, persimmon cv. Rojo Brillante, majorly located in the Valencian Community. The main postharvest disorders manifested by ‘Rojo Brillante’ persimmons are flesh browning, which is associated with mechanical damage and chilling injury displayed after low-temperature storage. Previous research has determined the postharvest conditions that lead fruit to develop such alterations. However, the biochemical process behind flesh browning and chilling injury disorders is still unknown.

Currently, there is special interest in introducing cultivars from other countries to broaden the varietal range. Besides, prolonging the fruit storage period to supply the markets according to the demand is one of the main challenges.

In this context, the present Thesis approached three main objectives: 1) Studying the biochemical process implied in the main physiological postharvest disorders manifested in persimmon fruits by focusing on changes in the fruit redox state; 2) Evaluating postharvest treatments to preserve fruit quality during cold storage; 3) Assessing the physico-chemical and nutritional quality of persimmon cultivars introduced from other countries to increase the varietal range.

Biochemical, chromatographic and microstructural studies have revealed that flesh browning manifested by fruits submitted to mechanical damage after removing astringency is associated with a tannins oxidation process caused by a stress oxidative situation. A new flesh disorder, “pinkish bruising”, has been described on fruits submitted to mechanical impacts while showing high astringency levels. Sensitiveness to the flesh browning disorder has also been evaluated on different cultivars introduced from other countries.

The implication of the redox system in the chilling injury manifestation on ‘Rojo Brillante’ persimmon has been determined. Moreover, we described the changes in this system associated with chilling injury alleviation by 1-MCP treatment.

The effect of a controlled atmosphere based on 4-5% O₂ + N₂ to prolong the storage of cultivars 'Rojo Brillante' and 'Triumph' has been seen to strongly depend on variety. The results were highly positive on cultivar 'Triumph', in which the evaluated atmosphere extended the storage period up to 3 months.

The use of short-term high CO₂ treatments was another technology assayed to alleviate chilling injury in non-astringent cultivar 'Fuyu'. This treatment significantly reduced the main chilling injury symptom manifested by this cultivar, which is flesh gelling. This effect was related to cell structure preservation.

Recent studies have shown that ethyl formate treatment is highly effective for pest control of persimmon 'Fuyu'. However, this treatment induces fruit softening, which causes quality loss. This Thesis revealed that ethyl formate treatment induces the activity of ethylene synthesis-related genes and that flesh softening is mediated by this hormone. It also demonstrated that by applying 1-MPC pretreatment, fruit softening associated with ethyl formate can be controlled. Therefore, the combined use of both treatments is seen as a potential treatment to disinfect persimmon fruits while preserving quality.

Finally, the study of ten cultivars introduced from other countries helped select the most interesting cultivars to broaden the varietal range according to their maturation date and their response to deastringency treatment. The main nutritional compounds of persimmon and how they are affected by CO₂ deastringency treatment are described.

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I. INTRODUCTION

I.1. ORIGIN, BOTANY AND MORPHOLOGY

Commercial persimmon fruits are derived from *Diospyros kaki* L. f. in the family Ebanaceae. It originated from China (with production records dating back to over 3.000 years) and was introduced into Japan and Europe in the 7th and 17th centuries, respectively. Other *Diospyros* species include *Diospyros lotus* L. f and *Diospyros Virginiana* L., but currently these are of interest as rootstocks (Woolf & Ben-Arie, 2011).

Botanically speaking, persimmon is a berry that consists in a rather homogeneous parenchymatous pericarp surrounded by a thin fragile skin. There are hundreds of different persimmon cultivars; its fruit shape varies from spherical to acorn, to flattened or squarish, while its weight can go from 30 g to more than 450 g depending on the cultivar (Fig. 1). The skin colour of fruit varies according to cultivar, from light yellowy-orange to dark orangey-red. Flesh colour also differs among cultivars as some persimmon cultivars depict a much more intense orange coloured flesh upon commercial harvest than others. The flesh made up of a dense cell structure may have large almond-shaped seeds in the inner section of all the approximately light carpels, but fruits will develop parthenocarpically. The calyx is a green four-lobed structure that surrounds the stem-end of fruit. Persimmon fruits are dependent on the calyx for gas exchange into fruit as there are no stomata or lenticels on fruit surfaces, which are covered with a waxy cuticle (Woolf & Ben-Arie 2011).



Fig. 1. External appearance of different cultivars of persimmon
Courtesy of Instituto Valenciano de Investigaciones Agrarias (IVIA)

An important feature of the some persimmon cultivars is the high soluble tannin content responsible for astringency. Astringency is the sensation that results when tannins bind salivary proteins and cause them to precipitate or aggregate, which leaves a rough "sandpapery" or dry sensation in the mouth.

According to the level of astringency upon harvest, persimmon cultivars can be classified into two general categories: astringent and non-astringent persimmons (also called 'sweet' persimmons) (Yonemori *et al.*, 2003). In both categories, there are cultivars in which fruit astringency is influenced by pollination (pollination variant) and cultivars whose fruits are not affected by pollination (pollination constant). Accordingly, persimmon fruits can be classified into four groups: 1) the Pollination Constant Non-Astringent (PCNA) group, which is not astringent and is with or without seeds, and persimmons can be eaten at harvest when they are crisp as apples; 2) the Pollination Variant Non-Astringent (PVNA) group, which is not astringent at harvest if fruits have seeds, and fruits are not edible when firm if they have been not pollinated; 3)

the Pollination Constant Astringent (PCA) group, which is always astringent when firm; 4) the Pollination Variant Astringent (PVA) group, which is also astringent if pollinated, and is not astringent only around seeds where they have dark tannin spots.

The persimmon cultivars that belong to the PCNA group have a low content of the soluble tannins responsible for astringency. Therefore, these cultivars can be consumed with high firmness after harvest. Nevertheless, the rest of the cultivars show a high soluble tannins content at harvest time; thus they must be subjected to postharvest deastringency treatments prior to their marketing, or otherwise they must be left on trees until they over-ripen, and can consequently be consumed as soft persimmons.

Table 1 shows the most important persimmon cultivars that belong to the four varietal groups which have already been characterised using molecular markers.

Table 1. List of some persimmon cultivars that belong to the four varietal groups

Cultivar	Group	Origin
'Fuyu'	PCNA	Japan
'Jiro'	PCNA	Japan
'Hana Fuyu'	PCNA	Japan
'O'goshō'	PCNA	Japan
'Izu'	PCNA	Japan
'Maekawa Jiro'	PCNA	Japan
'Cal Fuyu'	PCNA	Japan
'Koda Goshō'	PCNA	Japan
'Suruga'	PCNA	Japan
'Kaki Tipo'	PVNA	Italy
'Nishimura Wase'	PVNA	Japan
'Shogats'	PVNA	Japan
'Akagaki'	PVNA	Japan
'Amahyakume'	PVNA	Japan
'Giboshi'	PVNA	Japan
'Hachiya'	PCA	Japan
'Atago'	PCA	Japan
'Giombo'	PCA	Japan
'Yokono'	PCA	Japan
'Triumph'	PCA	Israel
'Saijyo'	PCA	Japan
'Aizumishirazu'	PVA	Japan
'Hiratanenashi'	PVA	Japan
'Tone Wase'	PVA	Japan
'Rojo Brillante'	PVA	Spain
'Sugitawase'	PVA	Japan

Source: Naval *et al.*, 2010; Giordani *et al.*, 2011.

I.2. PRODUCTION AND ECONOMIC VALUE

I.2.1. WORLDWIDE PRODUCTION

FAO statistical databases (2012) indicate worldwide production of 4.468.955 tonnes and 813.536 ha of cultivated area, as well as a continual increase in production (Table 2). China is the main producer, and shows an increase from 1 million tonnes in 1992 to over 3 million tonnes today. Its production is based on a wide range of cultivars with low yields, which are used for both fresh consumption and industrialisation, especially as dried persimmons.

In Japan, production has decreased in the last few years, while cultivated areas in Korea have significantly increased. Both countries mainly cultivate non-astringent cultivar 'Fuyu'. In Spain production comes close to 160.000 tonnes, and focuses on cultivars 'Rojo Brillante' and 'Triumph'. Brazil, with a similar production to Spain, has a large cultivated area of astringent cultivars, such as 'Rama Forte', 'Giombo' and 'Taubaté, sold in local markets. In the last few years, the production of non-astringent cultivars has also grown, such as 'Fuyu' and mainly intended for international markets. Persimmon production in Azerbaijan has doubled in the last decade and focuses on local astringent cultivars. Taiwan offers a similar range of cultivars to China. In Italy, persimmon production has lowered and centres on astringent cultivar 'Kaki Tipo', although cultivar 'Rojo Brillante' has appeared in recent years. Producer countries also include Uzbekistan, which focuses on local astringent cultivars and Israel, which produces mainly astringent cultivar 'Triumph'. Persimmons are a relatively new commercial crop for New Zealand, where production focuses on cultivar 'Fuyu' and is estimated at 2.000 tonnes of which around three quarters are exported.

Introduction

Table 2. Worldwide persimmon fruit production

Country	Production (Tonnes)
Worldwide	4.468.000
China	3.300.000
Korea	401.000
Japan	253.000
Spain*	160.000
Brazil	158.000
Azerbaijan*	140.000
Taiwan*	86.000
Italy*	47.000
Uzbekistan*	42.000
Israel*	31.000
New Zealand*	2.000

Source: FAOSTAT (2012) **estimated data*

I.2.2. THE IMPORTANCE OF THE PERSIMMON CROP IN THE MEDITERRANEAN REGION

Persimmon was introduced into the Mediterranean region by the end of the 19th century, first as an ornamental tree, and it was also appreciated for the quality of its wood. As a fruit tree, it was grown as isolated trees in gardens, family orchards or in small plantings, and their fruits were locally consumed. The species spreads along the Mediterranean coast, and coexists with citrus, fig and olive trees (Perucho, 2015).

Nowadays, Spain, Azerbaijan, Italy, Uzbekistan and Israel are the main Mediterranean producer countries, and Spain is the country with the largest and most rapidly expanding crop (Fig. 2).

Official Spanish statistics still consider the persimmon crop to form part of the group of "other fleshy fruits". Therefore, no official data about area and production exist. Probably, this is the reason why Spain does not appear in the FAO persimmon statistics. As mentioned above, Spanish production focuses on variety 'Rojo Brillante', with over 150.000 tonnes, which is produced mainly in the province of Valencia, followed by variety 'Triumph' with around 12.000 tonnes, produced mainly in Andalusia (south Spain).

Regarding Azerbaijan and Uzbekistan, production is based mainly on local astringent varieties that are exported mostly to Russia. An estimated 200.000 tonnes of persimmon are exported to the Russian market every year from former Soviet countries.

The main regions that grow the persimmon crop in Italy are Campania and Emilia-Romagna, where variety 'Kaki Tipo' represents around 90% of total production. Increase market competition by other fruits during the same season, such as apples, pears, table grapes and citrus, and the always very high marketing cost from many hand operations, are the reasons for the decrease noted in recent years. Besides there is another cause: competition of Spanish exports due to high quality cultivar 'Rojo Brillante'.

Israel extended the persimmon crop in the 1970s by introducing postharvest technology to remove astringency and applying cold storage. Nowadays, the persimmon production in Israel is around 31.000 tonnes and the most important variety (90% of the total) is 'Triumph', which is

commercialised under the commercial name of 'Sharon' or 'Sharoni' (LLácer & Badenes, 2002).

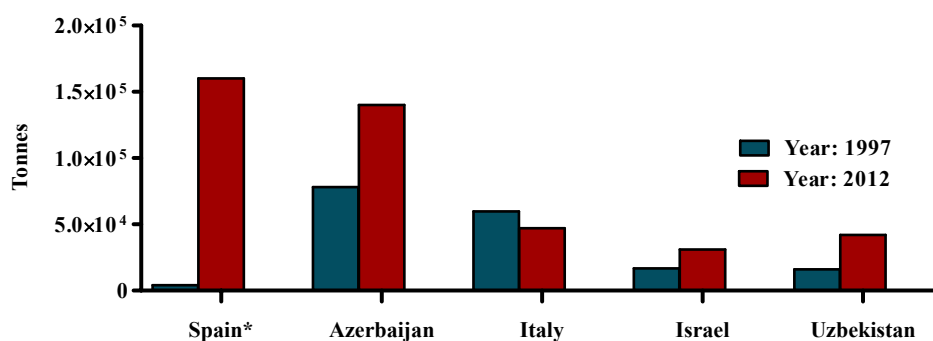


Fig. 2. Evolution of persimmon fruit production in Mediterranean countries from 1997 to 2012. Source: FAOSTAT (2012); *Perucho (2015)

Other countries such as Turkey, Greece, Egypt, Portugal and Morocco still generate very small production volumes, which are destined mainly to the domestic market. Turkey and Greece are introducing non-astringent persimmon varieties, such as 'Hana Fuyu' and 'O'gosh'. In Portugal, production is based on isolated species or those interspersed with other tree species. The most important variety is 'Coroa de Rei', which is astringent. 'Triumph', 'Hana Fuyu', 'O'gosh' are also grown. The persimmon crop from these countries shows a clear trend towards introducing non-astringent cultivars, although they are all starting to harvest 'Rojo Brillante' given its commercial success in European markets.

Other European countries also grown persimmons, but their production is under 2.000 tonnes per year; Cyprus and Slovenia grow varieties 'Kaki Tipo' and 'Lycopersicon', while Montenegro and Macedonia grow variety 'Costata' (Bellini & Giordani, 2005).

I.2.3. EVOLUTION OF THE PERSIMMON CROP IN THE VALENCIAN COMMUNITY. IMPORTANCE OF VARIETY ‘ROJO BRILLANTE’

In the Valencian Community, persimmon production over the last 20 years has exponentially increased (Fig. 3). The vast expansion of this crop has been due to diffusing astringent cultivar ‘Rojo Brillante’. Since 2002 to the present-day, the cultivated area of persimmon ‘Rojo Brillante’ has increased 6-fold, from an area with a little over 2.000 hectares to one that exceeds 13.000 hectares (Perucho, 2015).

The origin of the variety ‘Rojo Brillante’ is not totally clear. The most accepted hypothesis is that it comes from a bud mutation from variety ‘Cristalino’ which occurred in the ‘Ribera del Xúquer’ area of the Valencian Community in the 1940s. The new mutated cultivar has been grown along with other traditional varieties for many years. However, persimmon was not a commercial crop at that time. In the 1970s, the ‘Agriculture Extension Service’ in Carlet (province of Valencia) did a survey and selected persimmon cultivars in the area. From this survey, cultivar ‘Rojo Brillante’ was considered an outstanding cultivar. This cultivar is very productive with very large fruits that are attractive given their pleasant shape and colour, and their good flavour and aroma.

Due to its astringency, ‘Rojo Brillante’ was traditionally consumed over-ripened as soft persimmon; thus its fruits badly support handling and transport. The optimisation of techniques to remove fruit astringency with no loss of firmness was a substantial improvement in fruit marketing and exporting. This fact has been the main cause of persimmon crop expansion in the last few years. This crop replaced the apricot and Japanese plum plantations affected by the Sharka virus in this area (Llácer & Badenes, 2002).

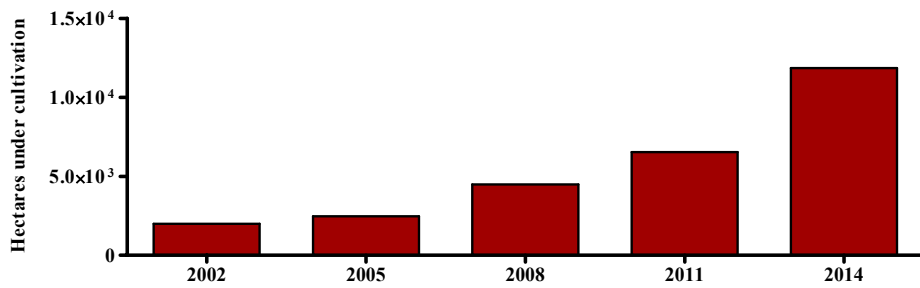


Fig. 3. Evolution of the cultivated area in the Valencian Community over the last few years.
Source: ESYRCE (2014)

Rapidly expanding ‘Rojo Brillante’ production, especially in the Ribera del Xúquer area, led to the Council Regulator of the Denomination of Origin (CRDO) ‘Kaki Ribera del Xúquer’ in 1997. The aim of this institution is to guarantee the quality and origin of persimmon ‘Rojo Brillante’ from this area.

The high fruit quality of this cultivar, optimisation of the techniques to remove astringency without losing firmness, together with the marketing campaigns carried out by the CRDO ‘Kaki Ribera del Xúquer’, have allowed exports to increase; currently around 80% of total persimmon production is destined to the international market (Fig. 4).

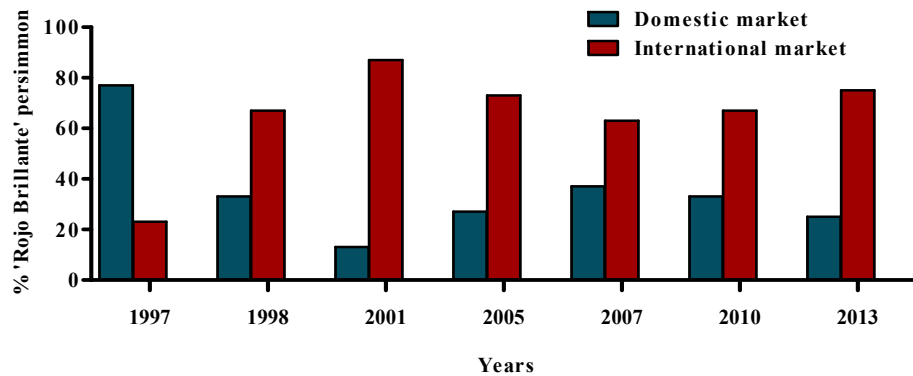


Fig. 4. Percentage of 'Rojo Brillante' persimmon destined to domestic and international market over the last few years.

Source: Perucho (2015)

Nowadays, in the Spanish Mediterranean, persimmon crop production focuses on a single variety, 'Rojo Brillante', with a limited harvested period (October to December). Moreover, a crop based on a monovarietal culture implies several commercial risks, which can compromise the future of the crop. Therefore, the main current challenges are to provide tools to extend the 'Rojo Brillante' commercial period and to introduce new varieties that broaden the varietal range.

In the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia), a germplasm bank was established in 2002 in order to collect cultivars of different origins and to evaluate their agronomic behaviour under agroclimatic Mediterranean conditions (Naval *et al.*, 2010). Besides pomology approaches, postharvest studies must be conducted to choose those cultivars whose quality, harvest time and postharvest life are demanded by the market.

I.3. FRUIT DEVELOPMENT AND MATURITY

I.3.1. PHYSICOCHEMICAL CHANGES DURING FRUIT GROWTH AND MATURATION

Persimmon fruits have been shown to follow a double sigmoid growth curve that consists in two rapid growth stages, stage I and stage III, which are separated by a period of slow growth (stage II) (Sugiura *et al.*, 1991).

Persimmon fruits are categorised as climacteric fruit, which means they are very sensitive to exogenous ethylene exposure and are induced to ripen with autocatalytic ethylene production by exposure to exogenous ethylene. They produce a small, but significant, amount of ethylene during the ripening period (Wills *et al.*, 1998; Kubo *et al.*, 2003; Besada *et al.*, 2010a). In the last decade, several molecular studies have demonstrated the involvement of the ethylene biosynthetic pathway and signal transduction in persimmon fruit ripening (Zheng *et al.*, 2005; Ortiz *et al.*, 2006; Pang *et al.*, 2007).

The external skin colour of this fruit is the index currently used as the non-destructive index for harvesting persimmons. The colour evolution displayed by persimmon cultivars comes from immature fruit that has a homogenous green skin to fruit with its characteristic orange or orange-reddish tones in the commercial maturity stage.

The colour changes that occur during the maturation process run in parallel to gradual flesh firmness loss. This fruit softening is related to microstructural changes in flesh. As maturity advances, parenchyma progressively degrades, less swollen and more deformed cells are observed, and cell walls and membranes degrade (Salvador *et al.*, 2007).

Other chemical changes accompany fruit maturation that contribute to the ripe fruit taste, which include sugar accumulation, acidity and reduced soluble tannin. Predominant sugars are glucose, fructose and sucrose, which increase through fruit development and reach a constant level prior to harvest maturity. During postharvest ripening, reducing sugars continue to increase due to both invertase activity (Zheng & Sugiura, 1990) and a concurrent drop in sucrose levels (Del Bubba *et al.*, 2009). Total acidity in persimmons is relatively low even in immature fruit, but does not change with ripening. Malic acid, which is the predominant organic acid, increases with maturity and is accompanied by

reduced citric acid (Senter *et al.*, 1991). Besides maturation and ripening are associated with reduced polyphenol content, which might lead to a lower antioxidant potential, although the increased carotenoid content associated with ripening is likely to raise it.

It is noteworthy that the maturity process can be delayed or advanced by preharvest treatments. An advance made in fruit maturity has been achieved by means of ethephon which when metabolised by the plant, is converted into ethylene, a potent regulator of plant growth and maturity. Hastening fruit maturity after ethephon applications has been reported in persimmon cv. Tone Wase (Kim *et al.*, 2004). Similarly gibberellin biosynthesis inhibitors, paclobutrazol and uniconazol, have been shown to advance the maturation of cv. Triumph (Ben-Arie *et al.*, 1997) and cv. Maekawa Jiro (Zilkah *et al.*, 2008), respectively. In contrast, the preharvest application of gibberellic acid (GA₃) when fruits are breaking colour delayed the harvesting of some persimmon cultivars, such as 'Triumph' (Agustí *et al.*, 2003), 'Fuyu' (Danieli *et al.*, 2002) and 'Hiratanenashi' (Nakano *et al.*, 1997). It must be taken into account that such treatments, which are used to modify harvest time, can also affect postharvest fruit evolutions. Different postharvest effects have been reported after these preharvest applications; the spraying of GA₃ delayed fruit ripening on trees and also lowered the postharvest fruit deterioration rate in cultivars like 'Triumph' or 'Rojo Brillante' (Ben-Arie *et al.*, 1997; Besada *et al.*, 2008a). Nevertheless, negative postharvest effects have been associated with paclobutrazol, which is applied to advance fruit ripening and has been shown to accelerate the postharvest deterioration rate of 'Triumph' fruit (Ben-Arie *et al.*, 1997).

I.3.2 CHANGES IN THE SOLUBLE TANNINS RESPONSIBLE FOR ASTRINGENCY

Astringency has been defined by the American Society for Testing and Materials as “the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannin acids” (ASTM, 1995). The astringency perception caused by food rich in polyphenols, such as tannins, is fairly well understood. A large family of proline-rich proteins is found in human saliva (Hay & Oppenheim, 1974; Kaufman & Keller, 1979), which are thought to serve wetting, lubrication and

protection functions of the oral epithelium. When polyphenols are ingested they cross-link salivary proteins and lead to the formation of insoluble complexes. Protein-polyphenol complexes reduce salivary lubrication between various oral surfaces and result in the tactile sensation of astringency (Thorngate & Noble, 1995; Prinz & Lucas, 2000). Precipitated proteins are free to adhere to mucosa and dentition, where they can form a sticky residue. Both reduced lubrication and sticky residue must inevitably increase the coefficient of friction between mucosal surfaces, a condition that should change the quality of the tactile sensations perceived when surfaces rub against each another (Green, 1993).

As mentioned above, persimmon cultivars can be classified into two general categories, non-astringent and astringent at harvest, depending on the concentration of the soluble tannins present in fruit flesh. Although the fruits of both groups are very astringent when small and immature, the former lose astringency while they grow on trees (Besada & Salvador, 2011a). During growth and ripening, non-astringent cultivars show fewer soluble tannins at levels that are sensorially non-detectable, even before fruit colour breaking, while the fruits of astringent cultivars maintain a high content of soluble tannins, even when fully coloured. Values of soluble tannins that come close to 0.03% have been reported in non-astringent 'Jiro' and 'Harbiye' persimmons (Taira *et al.*, 1998; Candir *et al.*, 2009), while astringent cultivars such as 'Hiratanenashi', 'Rojo Brillante' or 'Tipo' have presented a content of soluble tannins of 0.5-1% (Taira *et al.*, 1998; Salvador *et al.*, 2007; Del Bubba *et al.*, 2009). Therefore, it is necessary to apply postharvest treatments to remove astringency prior to commercialising cultivars that are astringent at harvest.

Soluble tannins of persimmon fruits accumulate in the vacuoles of specialised 'tannin cells' (Gottreich & Blumenfeld, 1991; Yonemori *et al.*, 1997). In the astringent persimmon group, tannin cell development is continuous until late fruit growth stages, while tannin cell development stops at early stages of PCNA-type fruit growth. Early cessation of tannin cell development is thought to be the main cause of natural astringency loss in PCNA-type fruit since it can dilute the concentration of tannins in flesh as fruits grow (Yonemori & Matsushima, 1985; 1987). A stop in tannin cell development in PCNA cultivars has been related to the expression of the genes involved in flavonoid biosynthesis which, in early fruit development stages, are expressed at high levels in both the PCA and non-PCNA types, but decline to become undetectable in PCNA-type cultivars in late development stages, which coincides with the termination of tannin accumulation. In contrast, these genes

in PCA-type cultivars are expressed at high levels until the late fruit development stage, which coincides with a continuous tannin accumulation in fruits (Ikegami *et al.*, 2005a, 2005b).

In astringent persimmons, the vacuolar sap of tannin cells contains 10-12% (m/v) of tannins, while parenchyma cells contain only trace amounts of tannins and a higher content of soluble sugars than tannin cells (Yonemori *et al.*, 1997). When persimmons of astringent-type cultivars are eaten, tannin cells in flesh are crushed and soluble tannins are released in the mouth, which leads to the formation of insoluble complexes by a reaction with salivary proteins. There are major differences between astringent cultivars in both tannin cell size and soluble tannins content (Yonemori *et al.*, 2003). In this sense, while the soluble tannins content is 0.26% fw in the fruits of astringent persimmon cv. Giombo (Antoniolli *et al.*, 2000), it is as high as 2.3% fw in the fruits of astringent cv. Tsurunoko (Yamada *et al.*, 2002). Besides the cultivar, the concentration of soluble tannins is affected by the maturity stage of the fruit during harvest and lowers as fruits become more mature (Yamada *et al.*, 2002). For example, the soluble tannins content in astringent persimmon 'Rojo Brillante' varies from 0.6% to 0.4% fw from the beginning to the end of the harvest period (Salvador *et al.*, 2007).

It has been reported that soluble tannin concentrations in persimmon below 0.1% do not cause the astringency sensation when this fruit is eaten (Vidrih *et al.*, 1994). This threshold has been assumed in several later studies (Antoniolli *et al.*, 2000; Yamada *et al.*, 2002; Antoniolli *et al.*, 2003). However, a soluble tannins content in some persimmon cultivars as low as 0.06% can produce detectable astringency (Besada *et al.*, 2010a). It is important to note that the astringency sensation produced by food rich in polyphenols can be affected by different factors, such as the repetition of stimuli, sweetness (Lyman & Green, 1990) or presence of acids (Siebert & Chassy, 2004), and even by the individual saliva flow and composition (Kallithraka *et al.*, 2001; Horne *et al.*, 2002). Thus it is logical to think that the threshold of soluble tannins in persimmon fruits that leads to the perception of astringency is affected by the characteristics of each cultivar as well as by the maturity stage of the fruit.

I.3.3. QUALITY AT HARVEST AND PHYTONUTRIENTS

Persimmons are harvested in autumn. Proper handling at harvesting is essential to achieve good quality persimmons and to determine marketability and profit. The recommended procedure to follow when harvesting persimmons is to clip fruits from trees with small secateurs, and to leave the calyx and a short stem attached to fruits. It is possible to snap fruits from trees, but this requires skill and increases its susceptibility to injury and subsequent decays. Generally, two to three picks are required depending on the cultivar and seasonal conditions.

As mentioned above, external colour is the property used as the non-destructive index for harvesting persimmon, which is the case of many other commodities. Fruits need to be well developed and to display the characteristic cultivar colour before being harvested. The sugar content of the fruits that remain on trees until a good colour develops will be higher, and fruits will have good flavour and consistency after harvesting. Immature fruits do not soften evenly after harvest and may be partly astringent and generally lack flavour. Most persimmon cultivars are considered ready for harvest when they are fully orange/yellow to orange-red colour and present no visible green background (Besada & Salvador, 2011b)

Unlike other fruits, in which the soluble solids measurement can be used as a good indicator of fruit maturity, this measurement in persimmon includes not only sugars, but also soluble tannins; thus the value of soluble solids in PCNA cultivars is lower than in astringent types (PCA, PVA, PVNA) due to the higher soluble tannin content in astringent ones. So the soluble solids measurement can be considered a good indicator of fruit sweetness and maturity, but only in non-astringent cultivars (Ullio, 2003) since the soluble tannins level is low and does not interfere with measurement. It must be noted that the same cultivar may behave differently depending on the climatic conditions where it is cultivated. So for the 'Fuyu' cultivar, a soluble solid content of 15° Brix is recommended at harvesting in New South Wales, while 'Fuyu' persimmons can attain soluble solids of up to 18° Brix in Japan (Ullio, 2003). Colour maturity charts that link external colour with internal maturity are useful tools, which must be developed for each cultivar, and even for each growing area.

Flesh firmness at harvest plays a decisive role in preserving the quality of fruits during the postharvest period. Loss of firmness is an unavoidable fact that will occur sooner or later, depending on the conditions under which fruits are maintained during the postharvest period. Therefore, the optimum external colour of persimmon at harvesting must be decided on not only the basis of the specific cultivar, but also on postharvest management procedures (Besada & Salvador, 2011b).

Persimmons are considered a good source of readily available carbohydrates and a high content of bioactive compounds, such as tannins, polyphenols, steroids, dietary fibre, organic acids, minerals and carotenoids, which contribute to the high antioxidant potential of these fruits (Santos-Buelga & Scakbert, 2000). These phytonutrients are considered important dietary supplements to reduce degenerative human diseases due to the capacity of these compounds to lower blood pressure and platelet aggregation (Mallavadhani *et al.*, 1998; Rao & Rao, 2007; Giordani *et al.*, 2011). In fact in some countries like China, persimmon fruits, and also persimmon leaves, have been traditionally used for many medical purposes; treating coughs, hypertension, dyspnoea, paralysis, frostbite, burns and bleeding (Matsuo & Ito, 1978). Recently, several research works have focused on persimmon fruit components, and related them to various physiological functions. Indeed it has been demonstrated that persimmons possess hypolipidemic and antioxidant properties, which are attributed to their water-soluble dietary fibre, carotenoids and polyphenols (Gorinstein *et al.*, 1998), with persimmon phenols being mainly responsible for the antioxidant effect of this fruit (Gorinstein *et al.*, 2011). Persimmon peel has also been shown to be a valuable source of antioxidants which, under diabetic conditions, can reduce the oxidative stress induced by hyperglycaemia (Yokozawa *et al.*, 2007).

Although data in the literature on phytonutrients in persimmon are widely available, they are affected by a number of variability sources, such as ripeness stage and analytical methods, which make it hard to obtain reliable and comparable results.

Varietal differences among persimmons are an aspect that should be addressed under the same agronomical conditions. In addition, the future studies should be performed on fruits in the “ready-to-eat” ripening stage, following general consumer preferences and nutritional purposes (Giordani *et al.*, 2011). Furthermore, since the destringency treatments are applied to astringent

cultivars prior to their marketing, it would be interesting to know the effect of this process from the physiological point of view, as well as the influence of the process on the phytonutrient profile.

I.4. POSTHARVEST PHYSIOLOGY AND TECHNOLOGY

After harvest fruits are subjected to continuous changes, some of which are desirable, and may require specific treatments and techniques to promote them, while many other are undesirable and require treatments to delay and minimise their incidence and severity. Although none changes in fresh fruits can be stopped, many can be delayed (Kader, 2002).

In the specific case of persimmon, the postharvest treatment to remove astringency is required for fruits of astringent cultivars before they are commercialised. Different methods are applied according to the cultivar and market preferences.

Fruit storage is also a common postharvest practice to help supply markets according to their demands. Depending on the storage period in question, as well as storage temperature and cultivar, different postharvest technologies are routinely applied to preserve fruit quality. When pests and fungal diseases are a problem, different postharvest procedures should be applied for their control to minimise economic loss.

Although persimmons do not require specific handling procedures other than usual grading, sizing and packaging, which have been adapted to final market requirements, we must take into account that persimmons must be gently handled to avoid mechanical impacts as they can cause physiological disorders.

I.4.1. REMOVING ASTRINGENCY

Astringent cultivars must be treated to remove astringency. Different methods have been used to this end, including ethylene or ethephon treatment, alcohol treatment, nitrogen or carbon dioxide gas treatment, or warm water treatment.

Treatments with ethylene (10ppm at 20°C) or ethephon (50-500ppm) are effective at removing astringency (Park *et al.*, 2003; Crisosto *et al.*, 2009). However, these treatments promote ripening and excessive fruit softening, which make the handling and commercialisation of persimmons very difficult.

Treatments based on maintaining fruits under anaerobic conditions or exposed to products that enhance anaerobic respiration have been introduced in order to remove astringency, while preserving fruit firmness. Methods such as exposing fruits to alcohol, CO₂, N₂, or warm water have proven effective to remove astringency and to extend persimmon shelf-life compared to overripe fruits. Numerous reports have indicated that the deastringency rate (decrease in soluble tannins) by using either of these treatments is related to acetaldehyde accumulation in flesh (Matsuo & Ito, 1978; Sugiura & Tomana, 1983; Pesis *et al.*, 1988; Taira *et al.*, 1989). The soluble tannins (water-soluble proanthocyanidins) of persimmon responsible for astringency are polymerised by acetaldehyde produced under anaerobic conditions to form insoluble compounds, which are non-astringent (Taira *et al.*, 1997). This mechanism has been investigated *in vitro* under mild conditions (pH 6-8) and has shown that persimmon tannins react with acetaldehyde in a relatively short time to become a gel (Matsuo & Ito, 1982). *In vivo* experiments have demonstrated that the soluble tannins responsible for the astringent character of persimmon fruits form an insoluble non-astringent product by a direct reaction with acetaldehyde (Matsuo *et al.*, 1991).

Acetaldehyde is known to be generated *in situ* by the oxidation of endogenous or exogenous ethanol, and by decarboxylation of pyruvic acid. Thus acetaldehyde accumulation in fruits can be achieved by submitting fruits to anaerobic conditions or by exposure to exogenous ethanol; piruvate decarboxylase and alcohol dehydrogenase are key enzymes that lead to acetaldehyde accumulation (Fig. 5).

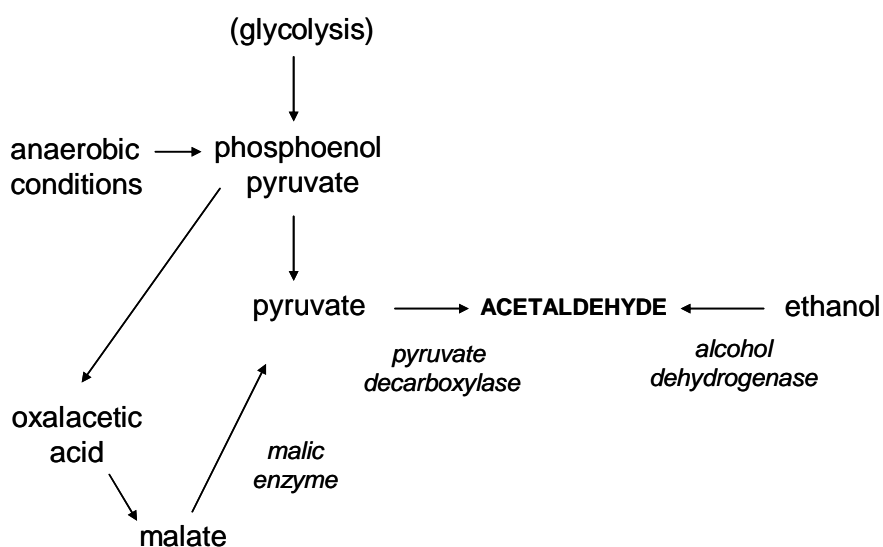


Fig. 5. Metabolic pathways for acetaldehyde production under anaerobic conditions and with external ethanol exposure (Adapted from Yamada *et al.*, 2002)

Acetaldehyde reacts with C-8 or C-6 of proanthocyanidin A-rings, and connects two proanthocyanidin molecules to result in their insolubilization and reduced astringency (Fig. 6). The covalent bonding of acetaldehyde in insolubilised proanthocyanidins has been supported by thiol degradation applied directly to the plant debris of fruits previously treated with ethanol under anaerobic conditions, which afforded bishioethers of flavan-3-ol acetaldehyde adducts (Tanaka *et al.*, 1994; 2010).

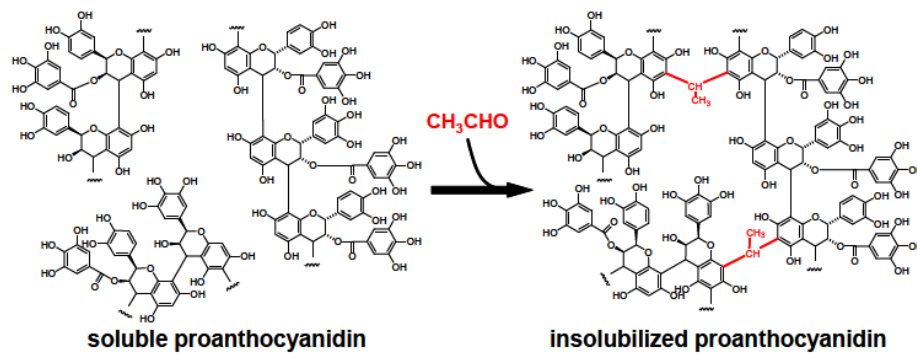


Fig. 6. Insolubilization of proanthocyanidins in persimmon fruits (Adapted from Tanaka *et al.*, 2010)

The precipitation of tannins induced by increased acetaldehyde production is reflected in the microstructure of flesh by the appearance of an insoluble material inside the vacuoles of some tannic cells (Salvador *et al.*, 2007).

Comparative studies into the effectiveness of such astringency removal methods have been conducted in many persimmon cultivars. Astringency can be removed successfully from several persimmon cultivars by the postharvest application of CO_2 - or N_2 -enriched atmospheres. In both N_2 and CO_2 treatments, the higher the concentration and the longer the exposure time, the faster the astringency decline rate. The drop in the level of soluble tannins in flesh parallels the acetaldehyde accumulation level in flesh with each treatment. The factor that limits the further extension of an enriched atmosphere treatment is reduced fruit firmness (Arnal & Del Río, 2003; Ahmed & Sobieh, 2007). While in some cultivars CO_2 treatment has proven more effective than N_2 (Zavrtanik *et al.*, 1999; Arnal & Del Río, 2003), in other cultivars N_2 treatments have been more effective and faster than CO_2 treatment in attaining a high taste score (Ahmed & Sobieh, 2007).

Another method of submitting fruits to anaerobic conditions is by dipping persimmons in warm water (around 25-40°C) for about half a day. This treatment, known as warm water treatment, has proven as effective at removing astringency as the treatment with high CO_2 concentrations (Taira *et al.*, 1989).

Introduction

However from the logistical point of view, this method is not normally used by industry.

The effectiveness of treatment using alcohol (ethanol vapour at 25%-35%) at removing astringency has also been widely studied. During this process, the acetaldehyde needed to insolubilise soluble tannins is produced by alcohol dehydrogenase directly from ethanol (Taira *et al.*, 1989, 1992b; Yamada *et al.*, 2002). As in other deastringency treatments, cultivar and harvest maturity are factors that affect the deastringency rate accomplished by ethanol application. The more immature the fruits and the higher the ethanol concentration, the faster the drop in soluble tannin content. The fact that astringency removal occurs more easily in younger fruits than in more mature ones can be due mainly to a more active ethanol conversion, which is taken into fruits during the treatment as acetaldehyde in flesh (Taira *et al.*, 1992a).

It is important to note that most studies that have compared the effectiveness of ethanol and CO₂ methods have shown that CO₂ treatment is significantly more effective at removing astringency than applying ethanol (Taira *et al.*, 1989, 1992b). The fact that soluble tannin content lowers more quickly after CO₂ treatment than after ethanol treatment is closely related to the acetaldehyde concentration in flesh; that is, the fruits treated with CO₂ accumulate acetaldehyde more rapidly than those treated with ethanol (Taira *et al.*, 1992a; Tanaka *et al.*, 1994; Yamada *et al.*, 2002). Tannins polymerise readily in those cultivars which rapidly accumulate acetaldehyde at high levels and the concentration of soluble tannins lowers slowly because of slow ethanol penetration through skin and/or slow alcohol metabolism into acetaldehyde (Taira *et al.*, 1992b; Tanaka *et al.*, 1994). To increase the deastringency process rate, methods that combine high levels of CO₂ and ethanol have been used (Taira *et al.*, 1992a).

With CO₂ treatment, which has been more extensively studied and used by industry, the deastringency process rate is influenced not only by treatment duration, but also by the maturity stage of fruits. Both factors influence the fruits degree of acetaldehyde production and, therefore, the astringency removal rate during and after treatment (Besada *et al.*, 2010a). Moreover, the higher the temperature during CO₂ treatment, the more astringency is removed (Ben-Arie & Sonego, 1993). An efficient commercial method used to remove astringency, while maintaining a high degree of fruit firmness, involves holding fruits in airtight chambers at 80-95% CO₂ at 20°C and 90% RH for 1-3 days, the optimal

duration depends on the cultivar, temperature and maturity stage (Taira *et al.*, 1989; Salvador *et al.*, 2007).

One point that should be considered is that low-oxygen atmospheres can induce oxidative burst in plants (Blokhina *et al.*, 2003), and fruit exposure to extreme anaerobic conditions, like 95% CO₂, will involve environmental stress and will lead to changes in the redox state of fruits. Although many studies have addressed the effect of deastringency treatment with CO₂ in the changes of physiological parameters, there is no information available about how CO₂ treatment can affect the redox status in which ROS (reactive oxygen species) levels and ROS scavenging enzymes are implied.

I.4.2. STORAGE

Storage of persimmon fruits is a common way of managing supply and to also prolong the commercial period at the end of the season. Freightin fruits to export markets can take a long time in certain cases, which implies having to maintain fruits under optimum transport conditions to preserve internal and external quality.

The most regularly applied system to prolong the postharvest life of perishable vegetables is refrigeration. Although the standard recommended storage temperature for persimmon fruits is 0°C (MacRae, 1987; Crisosto, 2004), some cultivars are sensitive to low temperatures, and they develop physiological disorders (chilling injury) when exposed at a temperature below a critical temperature (Testoni, 2002; MacRae, 1987; Collins & Tisdell, 1995; Arnal & Del Río, 2004).

I.4.2.1. Chilling injury

Chilling injury (CI) is a term used to describe the physiological damage that occurs in many plants and fruits, particularly those of tropical and subtropical origin, which is the case of persimmon fruits as a result of their exposure to low, but not freezing, temperatures (Jackman *et al.*, 1988). Sensitivity of persimmon fruits to CI is cultivar-dependent. Commercially

important cultivars, such as ‘Fuyu’ ‘Suruga’ or ‘Rojo Brillante’, are very susceptible to being CI sensitive, whereas other cultivars, such as ‘Triumph’ or ‘Hachiya,’ display less susceptibility to this disorder.

Although CI symptoms can vary depending on the cultivar, firmness disorders are reported as one of the main CI manifestations in all sensitive cultivars. So in cv. Fuyu, CI is initially expressed in the form of a flesh gelling, and is then later expressed by fruit darkening and increased skin transparency through which the characteristic gel may be seen (MacRae, 1987). In cv. Suruga and cv. Rojo Brillante, the main CI symptom is major loss of firmness (Collins & Tisdell, 1995; Arnal & Del Río, 2004). Other symptoms which have been associated with CI development, are loss of fruit flavour, sweetness, juiciness, titratable acidity or colour mottling, and skin translucence in cv. Fuyu (MacRae, 1987; Woolf *et al.*, 1997a), increasing °Brix and decreasing B-values in cv. Suruga (Collins & Tisdell, 1995), or compacted flesh areas and internal browning in ‘Rojo Brillante’ persimmons (Salvador *et al.*, 2005). During storage periods that are not overly long, the above-described CI symptoms usually appear when transferring fruits to places with higher temperatures (shelf-life). Yet during prolonged storage, these symptoms can eventually appear during cold storage (MacRae, 1987). Manifestation of CI depends not only on storage duration, but also on factors like storage temperature, cultivar sensitivity and even the fruit maturity stage; cultivars such as ‘Rojo Brillante’ (Salvador *et al.*, 2005, 2006) and ‘Fuyu’ (Krammes *et al.*, 2006) have shown a higher CI incidence when fruits are picked in early maturity stages.

Since CI manifestation can lead to severe loss in the postharvest fruit quality, several research works have focused on this disorder. Different studies have been carried out to understand the reasons for CI manifestation, and various methods have been tested with which to alleviate symptom development and to prolong storage periods.

CI manifestation has been mainly related to changes in cellular structure. Accelerated cell wall solubilisation of chilling-injured fruits has been reported in persimmon cv. Fuyu (Grant *et al.*, 1992). This fact has been corroborated in ‘Rojo Brillante’ persimmon, in which a microstructural study of flesh showed that softening, as a CI symptom, was the result of cell wall material degradation with loss of intercellular adhesion (Pérez-Munuera *et al.*, 2009). Likewise, Luo & Xi (2005) reported that it was not possible normally dissolution of the primary cell wall and the middle lamella in chilling-injured fruits when

transferred to normal temperatures after cold storage. Furthermore, CI manifestation and its alleviation by different treatments have been related to changes in both ROS level and the activity of oxidative system enzymes in persimmon (Zhang *et al.*, 2010) and in other fruits (Sala, 1998; Wang *et al.*, 2005).

Postharvest treatments to alleviate CI manifestation include pre-storage heat treatments (hot water and hot air treatments), manipulation of storage conditions (controlled and modified atmosphere storage) and exogenous chemical treatments (1-methylcyclopropene). As described in the following sections, these treatments have been shown to delay CI symptoms and to prolong the cold storage of persimmon fruits. Some have also been shown to have positive effects by prolonging the shelf-life period at moderate temperatures.

I.4.2.2. Treatments to alleviate chilling injury

1-Methylcyclopropene

1-Methylcyclopropene (1-MCP), an inhibitor of ethylene perception, has been shown to reduce CI symptoms in a large number of persimmon cultivars. Its recent availability has resulted in an explosion of research into its effects on fruits and vegetables, both as a tool to further investigate the role of ethylene in ripening and senescence, and as a commercial technology to improve product quality maintenance. 1-MCP is a non-toxic antagonist of the ethylene hormone that binds to the ethylene receptor after treatment (Sisler & Serek 1997). The success of 1-MCP treatment depends on its concentration, and on the temperature and duration of the application (Blankenship & Dole, 2003). Its efficacy also depends on the commodity, the cultivar, the harvest maturity and storage conditions (Watkins, 2008).

The effect of this compound on the postharvest life of persimmon fruits at both shelf-life temperatures and low temperatures has been studied in recent years. Due to the cultivar-dependent response to 1-MCP, its effect on each cultivar must be studied individually.

When applied at a concentration of 100-1000 nL L⁻¹, 1-MCP treatment delays the softening and colour evolution of persimmon fruits during their shelf-life. This effect has been observed in astringent cultivars ‘Tone Wase’ and ‘Saijo’ (Harima *et al.*, 2003), ‘Hiratanenashi’ (Kubo *et al.*, 2003), ‘Rojo Brillante’ (Salvador *et al.*, 2004) and ‘Triumph’ (Tsviling *et al.*, 2003), and in many non-astringent cultivars, like ‘Nathanzy’ (Ramin, 2008), ‘Rendaiji’ (Ortiz *et al.*, 2005), ‘Matsumotowase-fuyu’ (Niikawa *et al.*, 2005), Bianhua (Luo, 2004), ‘Qiandaowuhe’ (Luo, 2007) and ‘Fuyu’ (Fang *et al.*, 2009), among others. Although the extent of the effect of 1-MCP treatment clearly depends on the cultivar, in most cases the shelf-life period can at least double by applying the optimum 1-MCP concentration.

As mentioned above, the response of fruits to 1-MCP may be affected by the maturity stage at harvesting. This is the case of persimmon cv. Saijo, in which 1-MCP treatment lowered the fruit softening rate in the fruits harvested in the early and middle stages of maturity, while there was very little effect on late-harvested fruits (Kurahashi *et al.*, 2005).

As regards the optimum moment to apply treatment, fruits should be treated as soon as possible after harvesting. Delaying the postharvest 1-MCP treatment by up to 12 h might be acceptable in commercial practice. In astringent cultivars, 1-MCP may be applied during the treatment used to remove astringency by means of high CO₂ concentrations (Harima *et al.*, 2003).

1-MCP extends the shelf-life of persimmon and this effect is related to diminished ethylene evolution and to a delay in the ethylene peak appearing, which is due to an inhibition of the activity of ethylene-forming enzyme 1-aminocyclopropane-1-carboxylic oxidase (ACO) (Dongxing *et al.*, 2004). More recently, it has been demonstrated that 1-MCP treatment of persimmon inhibits the gene expressions of the two key enzymes involved in ethylene biosynthesis, ACO and 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Liu *et al.*, 2009).

Inhibition of ethylene formation by 1-MCP leads, in turn, to the inhibition of the enzymes that largely depend on ethylene, which is the case of cell wall degrading enzymes involved in fruit firmness loss. In this way, inhibition of pectinmethylesterase (PME) and polygalacturonase (PG) enzymes in 1-MCP-treated persimmons have been related to delayed softening during shelf-life (Xu *et al.*, 2004; Luo, 2004, 2007; Luo & Xi, 2005; Niikawa *et al.*, 2005). 1-MCP

treatment has also been shown to delay the accumulation of the mRNAs implied in gene expressions of cell wall degrading enzymes, such as cellulase (Cel), PG and expansin (Kubo *et al.*, 2003).

1-MCP treatment has been shown to delay the CI symptoms of persimmon fruits during low temperature storage, and to alleviate softening and gelling, which are the main symptoms in chilling-sensitive cultivars, such as 'Rojo Brillante' (Salvador *et al.*, 2004), 'Fuyu' (Kim & Lee, 2005; Krammes *et al.*, 2006), or 'Youhou' (Zhang *et al.*, 2010). Softening, as a CI symptom, is the result of cell wall material degradation with loss of intercellular adhesion. 1-MCP treatment preserves not only the integrity of cell walls, but also the adhesion between adjacent cells (Pérez-Munuera *et al.*, 2009), and also reduces membrane permeability (Zhang *et al.*, 2010) throughout the cold storage, and when fruits are transferred to a shelf-life temperature.

In addition to the activity of cell wall-degrading enzymes, as mentioned above, the cell structure can also be disrupted as a result of peroxidation caused by excess reactive oxygen species (ROS). The ROS metabolism is controlled by an array of interrelated enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), which act concomitantly with non-enzymatic antioxidants. Studying CI alleviation as a result of applying 1-MCP has been widely addressed in many cultivars in changes in flesh structure. However, only a single report on 'Fuyu' persimmon that has approached CI and its alleviation has taken into account changes in the oxidative system (Zhang *et al.*, 2010). Since the CI manifestation differs among cultivars, further studies about different persimmon cultivars in which CI manifestation can differ from others should be conducted so as to clarify the involvement of the oxidative system in CI manifestation and its alleviation by 1-MCP.

Nowadays, it is becoming commonplace to apply 1-MCP treatment to prolong the postharvest life of some important commercial persimmon cultivars, especially those that are prone to CI. Moreover, the additive effects of 1-MCP applied in combination with pre- and postharvest treatments are promising to further extend the postharvest life of persimmon fruits.

Controlled and Modified Atmospheres

Among the postharvest factors that are involved in maintaining the quality and in extending the commercial life of fresh fruit and vegetables we find the modification of O₂, CO₂ and/or C₂H₄ concentrations in the atmosphere that surrounds the commodity to different levels from those found in air. This is referred to as controlled atmosphere (CA) or modified atmosphere (MA).

CA is generally applied in specialised storage chambers which allow the atmospheric composition to be constantly controlled. MA is the practice of modifying the internal atmosphere composition of a package, which can be carried out by active or passive modification. In active modification, the target atmosphere is established by pulling a slight vacuum and replacing the atmosphere inside the package with the desired gas mixture. Additionally, absorbers may be included in the package, which scavenge O₂, CO₂ or ethylene to control the concentrations of these gases. A passive modification of the atmosphere is attained through the respiration of the commodity inside the package, and depends on the characteristics of both the commodity and the packaging material.

If used properly, MA and CA can supplement proper temperature management and can offer benefits, such as delayed senescence, reduced sensitivity to ethylene, alleviation of physiological disorders such as CI, or controlling insect and decay incidence. Such effects will depend on the fruit variety or cultivar, physiological state, atmospheric composition, storage temperature and storage time (Kader, 2002).

A great deal of research has been conducted in the last few years, which has aimed to identify the optimum atmospheric composition that will permit persimmons to be stored longer, while preserving the fruit quality characteristic of each cultivar. Successful CA and MA must maintain O₂ and CO₂ near to optimum levels in order to attain the beneficial effects of modifying the atmosphere without exceeding the limits of tolerance, which may mean an increased risk of physiological disorders and other detrimental effects. Although some general guidelines have been drawn up for persimmon fruits, which suggest the range of 3-5% O₂ and 5-8% CO₂ as being the appropriate atmospheric composition in which to improve their cold storage (Kader, 2002), this range may vary depending on the cultivar. Most of the research conducted into this aspect of persimmon fruits has focused on using MA bags, inside

which the desired atmosphere is generated passively during the cold storage of fruit. Good results have been obtained with polyethylene or low-density polyethylene bags, from 20 μm ('Fuyu' and 'Rama Forte') to 80 μm ('Fuyu') (Brackmann *et al.*, 1997; Cia *et al.*, 2006). Thus in several countries, such as New Zealand, Korea and Japan, 'Fuyu' persimmons are routinely stored in a modified atmosphere (MA) by sealing fruit in 60- μm polyethylene (PE) bags. This atmosphere delays and reduces CI symptoms and allows prolonged cold storage (MacRae, 1987; Kim & Lee, 2005). One of the main factors that limits longer storage life under MA conditions is the accumulation of ethanol and acetaldehyde, which cause off-flavours to develop, and may also result in tissue browning (Ben-Arie *et al.*, 1991).

Several research works have evaluated storing persimmon fruits in CA and have shown that this technology has the potential to improve fruit storage (Park, 1999; Donazzolo & Brackmann, 2002). As in MA storage, the optimum atmospheric composition will depend on the cultivar. Once again, internal browning and development of off-flavours are the main disorders that have been reported when O_2 and CO_2 concentrations are too low or high, respectively (Brackmann *et al.*, 2004; Park & Lee, 2008). Currently, storing persimmon fruits in a CA is not as common as MA packaging as MA makes it easier to maintain the desired atmosphere throughout the postharvest life; that is, from the time of packaging to when fruits reach final consumers.

Although, these techniques are effective for maintaining fruit quality and extending shelf-life, nowadays no specific optimal concentrations exist because they strongly depend on the maturity stage of fruits, storage temperature and the persimmon cultivar.

Pre-storage Heat Treatments

In the past few years, growing interest has been shown in using heat treatments as postharvest methods for controlling insect pests, preventing fungal rots and affecting the ripening or response of the commodity to temperature extremes. The response of fruits to heat treatments is usually cultivar-specific. In persimmon fruits, heat treatments have been evaluated mainly in 'Fuyu', a non-astringent cultivar, in which these treatments reduced the flesh gelling and flesh softening associated with CI. This effect has been observed when fruits

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were treated at temperatures of around 50°C by hot air treatments (HTs) (Woolf *et al.*, 1997a, 1997b) and hot water treatments (HWTs) before being cold-stored (Burmeister *et al.*, 1997; Lay-Yee *et al.*, 1997). Other positive effects of HWT, such as disinfestations, have been reported (Lay-Yee *et al.*, 1997). However, heat damage, mainly external and internal browning, has been associated with the application of heat treatments; the higher the HT temperatures and the longer the duration, the more damage done, which has led to browning levels that are likely to be commercially unacceptable (Woolf *et al.*, 1997a).

Among astringent cultivars, the heat treatments employed to control chilling injury were evaluated in 'Rojo Brillante' persimmon, in which the fruit response to HWTs has been shown to be heavily dependent on the maturity stage of fruits. HWTs (45-50°C for 20-40 min) applied to the fruits harvested in an early maturity stage alleviated CI, thus preserving fruit firmness, whereas the HWTs applied to fruits in more advanced maturity stages caused heat damage to fruits and did not reduce CI. In this case, not only were both external and internal browning reported as heat damage, but skin cracking was also observed. These physical disorders, associated with heat treatments, have been reported to be more marked when fruits were more mature and when HWT was carried out at higher temperatures and for longer times (Besada *et al.*, 2008b).

Based on the above-mentioned studies, heat treatments prove to be potential techniques to reduce CI in persimmon. In general, shorter heat treatments run at higher temperatures are required to alleviate CI. However, heat damage incidence may be a factor that limits heat treatment conditions (Burmeister *et al.*, 1997; Lay-Yee *et al.*, 1997; Besada *et al.*, 2008b). It should also be considered that its application may be an issue for companies from a logistical viewpoint. Given these limitations, nowadays this treatment is not usually applied by the persimmon industry.

1.4.3. PACKAGING OPERATIONS

Once harvested, fruits must be handled very carefully to avoid bruising as this is likely to result in marking, which becomes unsightly as fruits ripen. Harvested fruits should be placed in shallow containers with smooth or padded sides since persimmons are very prone to blemishes. Special care must be taken with cultivars with a pointed apex, such as 'Hachiya', to prevent them from causing any damage to other fruits.

Marketing requirements imply the polishing, grading and packaging of fruits. Persimmons can be polished manually with a cloth, but frequently this step is carried out by passing fruits through a packing-line, where they are cleaned by soft roller brushes. Since persimmon fruits are not usually waxed, it is important to maintain roller brushes in good condition to prevent natural wax from being removed. Then fruits are sized and packaged by hand, generally on single layer trays.

It is important to highlight that the bruising injuries to persimmon fruits during handling have been associated with faster firmness loss during cold storage (Lee *et al.*, 2005) and with an increase in both weight loss and skin darkness during shelf-life (Valentini *et al.*, 2009). For this reason, it is advisable to strive to minimise the number of fruit pieces dropped and height during equipment transitions, and to cushion unavoidable impacts with foam rubber and other materials, as persimmon fruits are very sensitive to mechanical impacts.

In astringent cultivar 'Rojo Brillante', mechanical damage suffered by fruits during packing-line operations has been determined as the main cause of flesh browning during the commercialisation period (Besada *et al.*, 2010b). Incidence of flesh browning is one of the main causes of postharvest loss for this cultivar. However, the mechanism for the manifestation of this disorder is still unknown.

In addition, the level of astringency of this cultivar when mechanically injured has been shown to influence incidence of browning. Thus fruits that have been treated with CO₂ to remove astringency are very susceptible, which indicates a possible role of tannins in this disorder (Besada *et al.*, 2012). Besides with environmental stresses, plants can lead to excessive ROS production, which can severely damage cell structures. The deastringency

treatment with high CO₂ concentrations, together with mechanical damage, can be considered a stressful environment for fruits since anaerobic atmospheres have been described as one of the environmental stresses that induces oxidative burst in plants (Blokhina *et al.*, 2003). Since no information is provided about the involvement of ROS and tannins in flesh browning manifestation, further studies should be addressed to elucidate this current issue, which leads to significant economic loss for persimmon industries.

I.4.4. CONTROLLING PATHOLOGICAL DISEASES AND INSECT PESTS

The incidence of pathological diseases and insect pests in persimmon fruits is highly dependent on the environmental conditions in the production area, the persimmon cultivar and disease control strategies.

It is noteworthy that in areas where persimmon fruits are a relatively new crop, cultivation has not been affected by severe incidence of pest and diseases. However, these issues are widespread in persimmon-growing areas with high production and wide cultivated areas, as in China, Korea and Japan, which result in severe economic loss. Furthermore in recent years, several pathological diseases have also been detected in the Mediterranean region, mainly due to postharvest pathogen *Alternaria alternata*. This is the case of Israel (Prusky *et al.*, 2001) and Spain (Palou *et al.*, 2012), where the planted surface of persimmon has greatly increased in the last few years and industries are using long-term storage.

Therefore, as pests and fungal diseases are a real problem in persimmon orchards and fruits are a susceptible host, knowledge of the pathogens and pests found in persimmon, and of postharvest handling procedures, is important for their control to minimise such economic loss.

I.4.4.1. Fungal diseases and their control

In general, postharvest decay can be caused in persimmon by the fungal inoculum that infects fruits through the injuries or microwounds located on any part of the skin (wound pathogens) or by an inoculum that infects flowers or young fruits in the field, which remains latent and develops after harvest (latent pathogens). Some pathogens are also able to infect stored persimmons by mycelial spread from infected fruit to adjacent healthy fruit, which causes “nests” of decay.

Several pathogens have been described to be causes of decay in persimmons, including species of *Alternaria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Neofusicoccum*, *Penicillium*, *Phacidiopycnis* and *Lasioidiplodia*. The limiting factor in their control is that formulations, which are widely used in most horticultural crops as a preharvest protective fungicide, are considered phytotoxic for persimmon fruits in most EU countries, and cannot therefore be applied.

The most important pathogen that affects persimmon fruits after harvest is *Alternaria alternata* (Fr.:Fr.) Keissl., which is the causal agent of black spot. Incidence of black spot is especially high in the ‘Triumph’ cultivar and has been described as the most economically important postharvest disease of this persimmon cultivar in countries like Israel (Prusky *et al.*, 2001). In other persimmon-producing areas, such as Brazil and Spain, its incidence has also been reported in cultivar ‘Fuyu’ (Park *et al.*, 1997, Cia *et al.*, 2003) and cultivar ‘Rojo Brillante’ (Palou *et al.*, 2009), respectively.

Alternaria alternata infects persimmon fruits through small wounds located under sepals of fruits and/or directly into the fruit cuticle (Prusky *et al.*, 1981). In general, *Alternaria* infections remain quiescent until harvest, when the disease slightly develops during storage at 0°C and spreads further during shelf-life. In years with heavy rain and/or high relative humidity before harvest, incidence of infection increases and small “active infections” are observed in wounded tissues before harvesting, which leads to a significantly increased incidence of decay during storage.

As they are extremely sensitive to black spot incidence, most of the research carried out into black spot control has focused on the ‘Triumph’ cultivar (Prusky *et al.*, 1981, 1997, 2001 and 2006; Kobilier *et al.*, 2011). As a

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result of this research, currently ‘Triumph’ persimmons are usually subjected to a chlorine dip treatment prior to storage at 0°C (Prusky *et al.*, 2001). Chlorine treatment is effective at controlling black spot in this cultivar for up to 2 months. However, decay incidence increases significantly the longer fruits are stored.

Although it seems that *Alternaria alternata* is repeatedly the most frequent pathogen to cause postharvest fruit decay, other causes of decay in persimmon with lower incidence and severity are: *Penicillium expansum* L. and *Cladosporium* spp, *Lasiodyplodia theobromae*, *Neofusicoccum mediterraneum* and *Neofusicoccum luteum*, in Spain (Palou *et al.*, 2013); *Botrytis cinerea* in New Zealand (Woolf *et al.*, 2008); *Mucor piriformis*, *Cladosporium gloeosporioides* in Korea and China (Zhang & Hu, 2004); *Rhizopus* in Brazil (Cia *et al.*, 2003); *Colletotrichum acutatum* in the United States (Williamson & Sutton, 2010); and *Phacidiopycnis washingtonensis* in Italy (Garibaldi *et al.*, 2010).

In any case, the type and incidence of fungal infections determine the potential economic losses caused by postharvest diseases every season and dictate the most adequate postharvest handling procedures to minimise such losses. This is even more important in Spain and other EU countries where no conventional chemical fungicides are currently permitted for postharvest persimmon treatment and disease control strategies rely on the integration of alternative non-polluting methods.

The application of emerging technologies to control physiological disorders and to extend postharvest fruit life, such as modified atmospheres or 1-MCP treatments, must be linked to the study and implementation of postharvest decay control systems since rot diseases may be limiting factors for long-term storage. In light of the above-mentioned results, and after more thorough studies, the integration of treatments may be a viable commercial practice for controlling postharvest diseases.

I.4.4.2. Insect pests and their control

Persimmons are a ready host to a wide range of insects, and become a significant problem for postharvest handling.

Fruit flies are one of the most important fruit pests that attack not only persimmon fruits, but also other cultivated species of high commercial value in subtropical and tropical areas in the world. Their control is difficult due to their relatively short life cycle. Sanitation recommendations include total removal and disposal of fruits from trees and orchard grounds after harvest. Each fruit-growing region has developed protocols to deal with this problem.

It is noteworthy that quarantine regulations in some persimmon-importing countries are very strict, with zero tolerance. Cool storage at below 1°C for 2 weeks kills all fly stages and is generally suitable for most cultivars.

Other important insects include thrips and scale insects, which can infest persimmon fruits or calyx. Where this occurs, it tends to result in indentations in fruits. Furthermore, various mites and other contaminating pests, such as spiders, earwigs, centipedes, slaters, may also be an issue because they can hide under the calyx or inside the calyx cavity. Thus they are difficult to detect and well protect from any preharvest insecticides and postharvest treatments.

In recent years, disinfestation procedures of horticultural products have focused on using methyl bromide as a postharvest fumigant for pest eradication. The advantages of methyl bromide are its low cost, easy application and broad spectrum. Nevertheless, methyl bromide was banned in developed countries in 2005 because of its ozone-depleting properties and risks to human health.

Thus the continuous ban of chemicals, such as methyl bromide, for pest control has increased commercial interest in other soft technologies that allow better market access for persimmon fruits. A number of postharvest disinfestation treatments (hot water treatments, controlled atmospheres, irradiation or radiofrequency heating) have been tested in persimmon fruits with limited success (Wheeler *et al.*, 1989; Mitcham *et al.*, 1997; Monzon *et al.*, 2007).

Restrictions on residues are increasing in many markets and new solutions are increasingly targeted as alternative solutions which leave no residues and do

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not damage the product. Therefore in recent years, industries have focused on using a Generally Recognised as Safe (GRAS) products, determined by the US Food and Drug Administration (US FDA), and such compounds are considered safe for use with human food.

Plant volatiles, such as ethyl formate (EF), have been shown to have insecticidal properties (Rohitha *et al.*, 1993). EF is considered a GRAS product and can potentially degrade to biogenic levels in the tissues of treated commodities before the product reaches the market. EF has been used for pest disinfestations in stored dried fruits since 1927 (Simmons & Fisher, 1945). EF is flammable and explosive when mixed with air at concentrations that kill pests, but formulations in CO₂ significantly reduce this risk; thus EF is available dissolved in liquid CO₂ in many countries under the trade name Vapormate[®] (Ryan & Bishop, 2003). More recently, EF applied as Vapormate[®] has been tested for its use in some fresh commodities such as persimmons, and proved effective against a wide range of surface pests: spider mite, western flower thrips, omnivorous leafroller, aphids, mealy bugs and black widow spiders (Simpson *et al.*, 2004; De lima, 2006; Simpson *et al.*, 2007; Van Epenhuijsen *et al.*, 2007; Finkelman *et al.*, 2010). Nevertheless with persimmons, this treatment can affect fruit quality (unpublished data). Therefore, future research should centre on developing reliable, non-toxic postharvest disinfestations treatment that is effective for pest control and for maintaining fruit quality. Integration of disinfestations and storage methods will be required to meet current market access requirements in each country.

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II. OBJECTIVES

GENERAL OBJECTIVE

To study the main physicochemical factors and nutritional components involved in the harvest and postharvest quality of persimmon fruit.

SPECIFIC OBJECTIVES

To investigate the involvement of oxidative stress in the main physiological postharvest disorders of persimmon fruit

To determine the effect of destringency treatment and mechanical damage on the fruit redox state and its involvement in flesh browning.

To evaluate the changes in the fruit redox system associated with chilling injury and its alleviation by 1-methylcyclopropene.

To evaluate postharvest treatments in order to preserve fruit quality during cold storage

To study the effect of controlled atmospheres on extending cold storage in different cultivars.

To evaluate the effectiveness of 1-methylcyclopropene to alleviate fruit softening induced by ethyl formate disinfection treatment.

To evaluate the physiological and nutritional quality of persimmon cultivars introduced from other countries in order to broaden varietal range

To characterise physiological maturity and to evaluate the response to destringency treatment of different cultivars.

To evaluate the influence of maturity stage and postharvest destringency treatment on the nutritional composition of different cultivars.

III. RESULTS AND DISCUSSION

***III.1. INVOLVEMENT OF OXIDATIVE
STRESS IN PHYSIOLOGICAL DISORDERS
OF PERSIMMON FRUITS***

CHAPTER I

Effect of CO₂ deastringency treatment on flesh disorders induced by mechanical damage in persimmon. Biochemical and microstructural studies

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Abstract

Manifestation of flesh browning while commercializing 'Rojo Brillante' persimmon is one of the main causes of postharvest loss. It is known that mechanical damage is a decisive factor for browning development and that astringent fruit is less sensitive to this disorder than fruit submitted to a CO₂ deastringency treatment under standard conditions (24 h, 95% CO₂, 20°C). However, there is no information available about the mechanism behind this alteration. In the present study, we evaluated the effect of treatment with high CO₂ concentrations applied for 0-40 h on the incidence of mechanical impact-induced flesh disorders using biochemical, chromatographic and microstructural techniques. Our results show that the longer the CO₂ exposure, the higher the incidence and the greater the severity browning. A deastringency treatment with CO₂ results in O₂⁻ accumulation in fruit, which is greater the longer treatment is. However, mechanical damage triggers the browning manifestation, resulting in the accumulation of both O₂⁻ and H₂O₂. In this oxidative stress state, which must be greater as higher the level of O₂⁻ previously accumulated in the deastringency treatment, insoluble tannins initially uncolor, undergo an oxidation process and turn red-brown, observed as flesh browning. Moreover, we identified a new disorder, "pinkish-bruising", which is manifested in astringent fruit. The mechanism of this alteration, also associated with mechanical damage, seems similar to that of browning, but the oxidation process would affect soluble tannins.

Keywords: Browning, Pinkish-Bruising, Reactive Oxygen Species, Tannins, Oxidative Stress, Tannins Oxidation

1. Introduction

Currently, the incidence of flesh browning is one of the main causes of postharvest loss of the 'Rojo Brillante' persimmon, which is the persimmon cultivar mainly cultivated in the Mediterranean region. The cause of this disorder, which appears during the commercialization period, remains unknown. It was recently reported that the mechanically damage that fruit are submitted to during packing operations may be a decisive factor of browning manifestation (Besada, Arnal, Salvador & Martínez-Jávega, 2010a).

As this cultivar belongs to the group of persimmon cultivars that are astringent at harvest, fruit are routinely submitted to a postharvest deastringency treatment based on the exposure of fruit to a high CO₂ concentration in order to remove astringency prior to commercialization (Salvador, Arnal, Besada, Larrea, Quiles & Pérez-Munuera, 2007). Persimmon astringency is due to the high concentration of soluble tannins present in fruit flesh. The effectiveness of CO₂ treatment to remove astringency is based on the insolubilization of tannins by the acetaldehyde generated during anaerobic respiration intermediating, which is triggered when fruit is exposed to a high CO₂ atmosphere (Matsuo & Ito, 1982; Matsuo, Ito & Ben-Arie, 1991). Application of treatment at 95-100% CO₂ for 24 h at 20°C has been established as optimal conditions to ensure the removal of astringency in 'Rojo Brillante' persimmon (Salvador et al., 2007; Besada, Salvador, Arnal & Martínez-Jávega, 2010b). Our former studies showed that fruit which have been previously submitted to the deastringency treatment (non astringent fruit) are susceptible to manifesting mechanical impact-induced browning, while astringent fruit are less susceptible to displaying this alteration after packing operations. Thus tannins have been suggested to be implied in the browning process (Besada, Salvador, Vázquez-Gutiérrez, Hernando & Pérez-Munuera, 2012).

One of the main causes of browning in fruit and vegetables is the oxidation and polymerization of phenolic compounds due to the activation of enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD). In persimmon fruit, the cause of external bruising has been poorly investigated. Lee, & Kim and Park (2005) reported no significant changes in lipid peroxidation, as expressed by malondialdehyde production, among bruised and unbruised persimmon cv. Fuyu; these authors also reported that although increased polyphenol oxidase activity appeared to be associated with the visual deterioration of bruised fruits, it could not be the only factor to influence the bruising manifestation. Similarly, our previous studies showed no

significant changes in PPO and PAL activity between browned and sound flesh of 'Rojo Brillante' persimmon. However, POD enzyme activity has been associated with mechanical damage and browning manifestation (Khademi, Salvador, Zamani & Besada, 2012).

Furthermore, reactive oxygen species (ROS) are produced as a normal plant cellular metabolism product. Various environmental stresses lead to excessive ROS production; such disturbances in the equilibrium between the production and scavenging of ROS bring about a sudden increase in intracellular ROS levels, which can severely damage cell structures (Gill & Tuteja, 2010; Sharma, Jha, Dubey & Pessarakli, 2012). Anaerobic atmospheres have been described as one of the environmental stresses that induces an oxidative burst in plants (Blokina, Chirkova, & Fagerstedt, 2001; Blokina, Virolainen & Fagerstedt, 2003). Moreover, changes in ROS levels have been related to fruit deterioration in association with mechanical damage of fruit like apricots and pears (De Martino, Vizovitis, Botondi, Bellincontro & Mencarelli, 2006; Li, Yan, Wang, Zhao, Cao & Jiang, 2010). There is no information about the effect of CO₂-deastringency treatment on the redox state of persimmon, and no studies have addressed the involvement of ROS in persimmon browning in association with mechanical damage.

This study aimed to investigate the implication of not only the deastringency treatment with high CO₂ concentrations, but also therefore of the level and form (soluble/insoluble) that tannins take in the mechanical-induced browning of persimmon fruit, as well as the involvement of ROS in this disorder. Microstructural techniques were used to describe browning alterations.

2. Materials and Methods

2.1. Vegetal material and experimental design

Persimmon (*Diospyros kaki* Thunb. cv. Rojo Brillante) fruit were harvested in l'Alcúdia (E Spain) at commercial maturity stage. The harvesting date was November 8, when fruit presented an External Colour Index=11 and firmness value of 44,9 N. After harvest, fruit were taken to the Instituto Valenciano de Investigaciones Agrarias (IVIA), where they were carefully selected for uniformity of size and color, and for lack of defects.

Thirty-six lots of 20 fruits were formed and one lot of fruit was used to determine the maturity stage at harvest. After harvest, fruit were exposed to CO₂ treatment (95% CO₂ at 20°C and 90% RH) for 0, 6, 12, 18, 24, 32 and 40 h (5 lots of 20 fruit per time). After each CO₂ treatment, one lot of fruit was directly analyzed. The remaining lots of fruit were submitted to mechanical packing (packing-line) or were carefully hand-packed. Finally, fruit were stored at 1°C or 15°C for 15 days. Lots of 20 fruits were used to study each postharvest condition.

CO₂ treatments were carried out in closed containers which contained 95% CO₂ at 20°C and 90% RH for the aforementioned times. These conditions were established by passing a stream of air containing 95% CO₂ through the containers.

After CO₂ treatment, the following analyses were carried out: soluble tannin content (ST); sensory evaluation of astringency; acetaldehyde concentration (AcH); ROS superoxide anion (O₂⁻) and peroxide (H₂O₂); microstructural studies by Scanning Electron Microscopy at Low Temperature (Cryo-SEM).

After evaluating storage, the incidence and severity of internal disorders and the superoxide anion (O₂⁻) and peroxide (H₂O₂) were analysed. Microstructural studies by Cryo-SEM and Light Microscopy (LM) were also performed. Besides, chromatographic techniques were used to determine the flavan-3-ol units forming tannins from flesh samples.

2.2. Fruit assessment

2.2.1. Assessment of flesh firmness and skin colour

Flesh firmness was evaluated with a Texturometer Instron Universal Machine, model 4301 (Instron Corp., Canton, MA, USA) using an 8-mm plunger. Fruit firmness values were an average of 20 fruit per treatment. The results were expressed as loads in Newtons (N) to break the flesh on each fruit on 180° sides after removing peel.

Fruit skin colour was evaluated using a Minolta Colorimeter (Model CR-300, Ramsey, NY, USA). The 'L', 'a', 'b' Hunter parameters were measured and the results were expressed as a External Colour Index (CI) = (1000a)/(Lb).

2.2.2. Assessment of soluble tannins and acetaldehyde

To determine soluble tannins and acetaldehyde concentration, lots of 15 fruit per treatment were divided into three samples and were cut into four longitudinal parts. Two opposite parts were sliced and frozen at -20°C to determine soluble tannins. The other two opposite fruit parts were placed in an electric juice extractor and the filtered juice was then employed to establish acetaldehyde concentration. Soluble tannins were evaluated by the Folin-Denis method (Taira, 1995); the results were expressed as a percentage of fresh weight. Three replicates of acetaldehyde concentration were measured per juice sample and were analyzed by headspace gas chromatography, as described by Salvador, Arnal, Monterde and Cuquerella (2004). The results were expressed as $\text{mg. } 100\text{mL}^{-1}$ of juice. Data were subjected to an analysis of variance, and multiple comparisons between means were determined by the least significant difference test ($P = 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA).

2.2.3. Assessment of total antioxidant capacity

The total antioxidant capacity was determined as the antiradical activity of methanolic extracts. It was spectrophotometrically tested by measuring the decrease in absorbance of the free radical DPPH, according to Brand-Williams, Cuvelier and Berset (1995). The decrease in absorbance at 515nm was monitored after 30 min when the reaction reached a plateau. The percentage of DPPH neutralized at the steady state (%Inhibition) was determined by using the following equation: $\% \text{ Inh} = ((\text{Abs B} - \text{Abs E}) / \text{Abs B}) * 100$, where Abs B and Abs E are the absorbance of the blank (B) and the extract (E) respectively. The DPPH inhibition percentages obtained were referred to mg of flesh.

2.2.4. Assessment of ROS

ROS species were detected *in vivo*. O_2^- production was analyzed by the method described by Doke (1983). Five flesh fruit discs were immersed in 10 ml of 0.01 M potassium phosphate buffer (pH 7.8) containing 0.05% nitroblue tetrazolium (NBT) (Sigma, St. Louis, MO, USA) for 2 h.

Hydrogen peroxide (H_2O_2) was visually detected in fruit discs using 3,3-diaminobenzidine (DAB) (Sigma) as a substrate by following the method described by Orozco-Cárdenas and Ryan (1999). Five green fruit discs were placed in 10 cm-diameter Petri dishes containing DAB solution (1 mg/ml, pH

3.8) for 2 h under light at 25°C. The assay was based on the instant polymerization of DAB (to form a reddish-brown complex that is stable in most solvents) when it comes into contact with H₂O₂.

2.2.5. Assessment of internal disorders

The incidence of internal disorders was evaluated as the percentage of affected fruit, while the severity of the disorder was evaluated on five scales as so: 0: absence 1: slight 2: moderate, 3: severe and 4: highly severe.

In order to obtain a unique value that reflects both the incidence and severity of each disorder, we used the following index, the Disorder Index= $\sum[(\text{Disorder severity}) \times (\text{number of fruit at each disorder severity})] / (4 \times \text{total number of fruit in the treatment})$.

2.2.6. Microstructural studies

Microstructural studies were performed by Cryo-Scanning Electron Microscopy (Cryo-SEM) and Light Microscopy (LM). For Cryo-SEM observation, cubes (3mm³) were cut from the equatorial area perpendicularly to the main axis of the persimmon flesh with a stainless steel cutter. These cubes were immersed in slush nitrogen (-210°C) and were then transferred to a cryo-stage (CT-1500 C from Oxford Instruments, Oxford, England) linked to a scanning electron microscope JEOLJSM5410 (JEOL, Tokyo, Japan) operating at a temperature below -130°C. Samples were cryofractured at -180°C, etched at -90°C and covered with gold (0.2 kPa, 40 mA). Microscopic observations were carried out at 15 kV and at a working distance of 15 mm.

For LM observation, hand-cut sections were obtained from the persimmon and examined under a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan) without being stained.

2.2.7. Chromatographic studies

Flavan-3-ol units forming tannins were determined on lyophilized flesh samples taken from astringent sound fruit (0h- CO₂ treatment plus hand packing), non astringent sound fruit (24h- CO₂ treatment plus hand packing), non astringent browned fruit (24h- CO₂ treatment plus mechanical packing) and fruit submitted to controlled oxidation (non astringent fruit + KO₂).

Thiolytic cleavage reactions and chromatographic techniques were applied to determine the proportion of flavan-3-ol units in the mentioned flesh samples. Tannins were extracted from flesh samples with 60% ethanol/water. After centrifugation, both supernatant (containing soluble tannins) and pellet (containing insoluble tannins) were submitted to thiol-degradation with mercaptoethanol-HCl at temperature of 70°C. Product from thiol-degradation was analyzed by means of HPLC-DAD according to Zhang and Lin (2008). Besides, in order to simulate a non-enzymatic oxidation process, 100mM KO₂ was added to lyophilized samples of non-astringent hand packed fruit one hour before starting tannins extraction.

2.2.8. Statistical analysis

Data were subjected to an analysis of variance, and multiple comparisons between means were determined by the least significant difference test ($P = 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA).

3. Results

3.1. Study of the physiological parameters related to astringency

Soluble tannin and acetaldehyde content are the main parameters relating to the astringency removal process when submitting fruit to high CO₂ concentrations. Therefore, we determined them both in the fruit submitted to different CO₂ treatments (0, 6, 12, 18, 24, 32 and 40 h). Besides, fruit astringency was also evaluated by a sensory panel.

The fruit not exposed to CO₂ (0 h treatment) showed a soluble tannins content of 0.6% fw (Supplementary Fig. S1A), which are habitual values at harvest in the 'Rojo Brillante' fruit at this maturity stage. The application of the 6 h CO₂ treatment led to a decrease in soluble tannins with values of 0.5% fw. A more marked reduction in the level of soluble tannins was observed when fruit were subjected to a 12-hour treatment, which led to values of 0.13% fw. When CO₂ treatment was performed for 18 h and 24 h, the soluble tannins content fell to values of 0.08 and 0.03% fw, respectively. Nevertheless, longer CO₂ exposures did not lead to soluble tannins to further decrease; CO₂ applications

lasting 32 h and 40 h resulted in a similar ST content as the 24-hour treatment: 0.03% fw (Supplementary Fig. S1A).

The sensory evaluation of fruit after different CO₂ treatments revealed a high degree of astringency in the fruit treated for 6 h, a moderate and slight level of astringency in those treated for 12 h and 18 h, respectively, while astringency was not detected in the fruit treated for 24 h or longer (32 h and 40 h). As expected, the fruit that were not submitted to CO₂ treatment (0h CO₂) was evaluated as intensively astringent (data not shown).

Supplementary Figure S1B shows the acetaldehyde concentration which accumulated in the fruit flesh after being submitted to CO₂ exposure for 0-40 h. The fruit that were not subjected to CO₂ treatment (0h CO₂) presented an acetaldehyde content close to 0.15 mg/100mL. When fruit were exposed to CO₂ between 6 h and 32 h, AcH accumulation increased the longer the CO₂ treatment lasted. So, the acetaldehyde level was 1 mg/100mL in the 6 hour-treated fruit, while the 32 hour-treated fruit gave values as high as 9 mg/100mL. However when treatment was prolonged to 40 h, the level of accumulated acetaldehyde in the fruit did not increase if compared to the 32 hour-treated fruit (values of 9 mg/100mL).

The total antioxidant capacity (TAC) of the fruit after being submitted to CO₂ exposure for 0-40 h is shown in Supplementary Figure S1C. The fruit not exposed to CO₂ (0 h treatment) showed TAC values close to 80% Inh/ mg fw. Exposure of the fruit to CO₂ for 6 h to 24 h resulted in a progressive loss of TAC, which was specially accused after exposures that lasted 12 h or longer. Fruit submitted to CO₂ treatment for 32 h and 40 h showed the same values of TAC than fruit treated for 24 h (20% Inh/ mg fw).

3.2. Incidence and severity of internal disorders after storage

The visual evaluation of fruit after storage revealed that, irrespectively of CO₂ treatment and storage temperature, hand-packed fruit did not manifest internal disorders (0% incidence of disorders). However, two different types of alterations were observed in the fruit submitted to mechanical packing: flesh browning, which is the initial aim of this study, but also another disorder which we named “pinkish-bruising”. Flesh browning was observed as large browned areas of flesh extending around the fruit (Supplementary Fig. S2A and S2B).

The pinkish-bruising disorder was seen as isolated areas of pulp in which the habitual orange tones turned pinkish (Supplementary Fig. S2C, S2D).

Figure 1 presents the percentage of the mechanically packed fruit affected by the pinkish-bruising and browning disorders after storage periods. In order to obtain a single value that reflects both incidence and severity, the disorder index was determined (Supplementary Table S1).

Irrespectively of storage temperature, 15°C or 1°C, no flesh browning was detected in the fruit that were not submitted to CO₂ treatment (0 hour-treatment) or in the fruit submitted to CO₂ treatment for 6 h. Exposure of fruit to CO₂ for 12 h followed by mechanical packing gave 10% and 40% of slightly browned fruit after storage at 15°C and 1°C respectively.

All the fruit treated with CO₂ for 18 h or longer (24h CO₂, 34h CO₂ and 40h CO₂) manifested browning; the intensity of this disorder was greater the longer the CO₂ exposure was. Moreover, the fruit stored at 1°C displayed more intense browning than that stored at a moderate temperature (15°C). Unlike the browning manifestation pattern observed, the incidence and severity of pinkish-bruising were higher the shorter the CO₂ exposure time was. So, 100% of 0h-treated fruit manifested pinkish-bruising, and this fruit showed the greatest alteration intensity. The longer the CO₂ exposure time became, the severity and incidence of this alteration diminished, and were not detected in the 32 h- and 40 h-treated fruit. Similarly to browning, the highest incidence and severity of pinkish-bruising were observed after storage at low temperature.

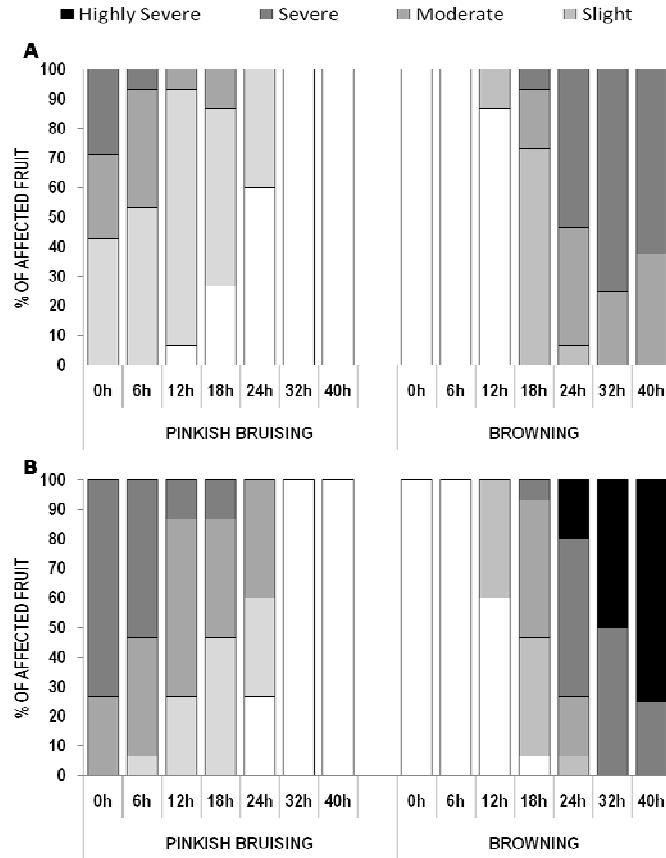


Fig. 1. Incidence and severity of pinkish-bruising and browning in persimmon cv. Rojo Brillante submitted to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 0, 6, 12, 18, 24, 32 or 40 h, plus mechanical packing, and then stored for 15 days at 15°C (A) or 1°C (B).

3.3. Correlation among physiological parameters after CO₂ treatment and internal disorders after storage

The physiological parameters relating to the level of astringency after different CO₂ exposures (soluble tannin content, acetaldehyde content and total antioxidant capacity) correlated with the Disorder Index (pinkish-bruising and browning), and are shown in Supplementary Table S2.

Both soluble tannin and acetaldehyde content correlated strongly with the manifestation of flesh alterations. Browning inversely correlated with soluble tannin content and positively correlated with acetaldehyde content, while pinkish-bruising positively correlated to soluble tannins content and inversely correlated with acetaldehyde content.

Total antioxidant capacity after CO₂ treatments descended in parallel to soluble tannins content. Therefore, it also correlated positively with the pinkish-bruising index and inversely with the browning manifestation.

3.4. Study of reactive oxygen species (ROS)

Reactive oxygen species, O₂⁻ and H₂O₂, were detected by *in vivo* staining with NBT and DAB. The reaction of O₂⁻ with NBT leads to blue staining, while a brown polymerization product is formed by the reaction of DAB with H₂O₂.

Figure 2 shows the results of the *in vivo* detection of O₂⁻ and H₂O₂ after exposing fruit to CO₂ from 0 h to 40 h. The level of O₂⁻ significantly increased with hours of CO₂ exposure; while no levels of O₂⁻ were detected in the fruit that were not submitted to CO₂ (0h- CO₂), NBT staining was low, but levels of O₂⁻ were detected in the 6 h-treated fruit. Indeed, the longer the CO₂ exposure time was, the higher the levels of accumulated O₂⁻ in fruit flesh, as revealed by blue staining.

When fruit were immersed in DAB, no brown staining was detected, irrespectively of CO₂ treatment duration. This indicates that no relevant changes in the H₂O₂ levels were associated with CO₂ exposure.

The *in vivo* detection of ROS in astringent (0h- CO₂) and non astringent fruit (24h CO₂) after the storage period at 1°C is shown in Figures 3A and 3B, respectively. In both cases, hand-packed (no manifesting disorders) and mechanically packing fruits (with flesh disorders) are presented. After the storage period, NBT staining revealed the appearance of detectable levels of O₂⁻ in the 0h CO₂ fruit associated with the pinkish-bruising manifestation (Fig. 3A4). No detectable levels of O₂⁻ were observed in the hand-packed fruit not treated with CO₂ (0h), in which no flesh disorders were observed (Fig. 3A3). A general increase in the level of O₂⁻ was observed in both hand-packed and mechanically packed fruit submitted to CO₂. Moreover in the mechanically

packed fruit, more intense blue staining was seen in flesh areas where browning was manifested (Fig. 3B7, 3B8) as compared to sound flesh areas (Fig. 3B5, 3B6).

The DAB staining of the different samples revealed H_2O_2 accumulation to be associated with mechanical impacts. Hence in both submitted fruit, but not to CO_2 , brown staining was detected in the flesh areas in which pinkish-bruising and browning disorders were manifested (Fig. 3A6, 3B11, 3B12). No staining was noted in the hand-packed fruit or in those areas of the mechanically packed fruit that remained sound (Fig. 3A5, 3B9, 3B10).

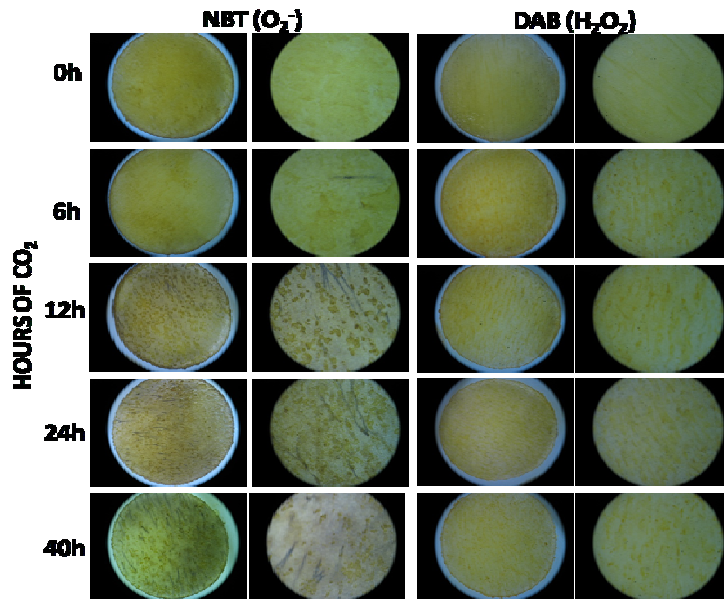


Fig. 2. In vivo detection of anion superoxide (O_2^-) and peroxide (H_2O_2) in flesh samples of persimmon cv. Rojo Brillante submitted to treatment with a high CO_2 concentration (95% CO_2 , 20°C, 90% H.R.) for 0, 6, 12, 24 or 40 h. Areas of O_2^- accumulation are indicated by the formation of a blue reaction product with NBT. Areas of H_2O_2 production are indicated by the formation of a brown reaction product with DAB.

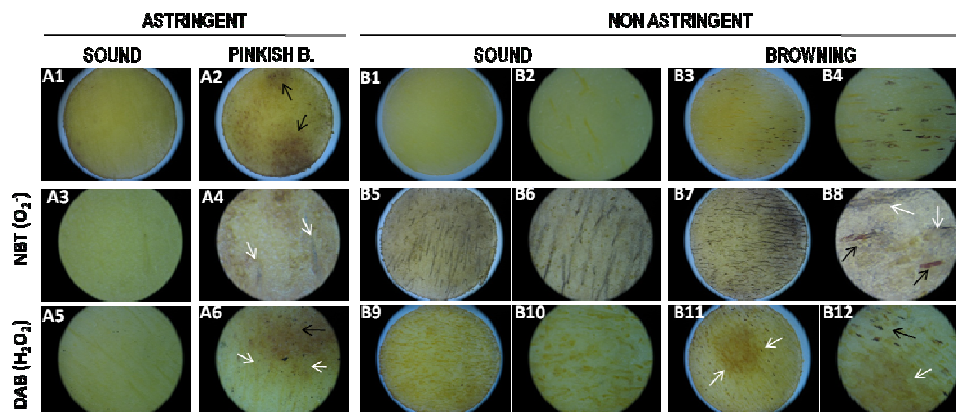


Fig. 3. In vivo detection of anion superoxide (O_2^-) and peroxide (H_2O_2) in flesh samples of persimmon cv. Rojo Brillante stored at $1^\circ C$ for 15 days. (A) astringent persimmon stored after being subjected to hand packing (with no flesh disorders-sound flesh) (A1, A3, A5) or mechanical packing (with pinkish-bruising) (A2, A4, A6). (B) non astringent persimmon (submitted to deastringency treatment for 24h) stored after being subjected to hand packed (with no flesh disorders-sound flesh) (B1, B2, B5, B6, B9, B10) or mechanical packing (with browning) (B3, B4, B7, B8, B11, B12). O_2^- accumulation is indicated by the formation of a blue reaction product with NBT. H_2O_2 production is indicated by the formation of a brown reaction product with DAB. Aspect of flesh samples before NBT and DAB addition is shown in A1, A2, B1, B2, B3, B4. Black arrows indicate pinkish bruising (A) or browning (B) manifestation; white narrows denote NBT and DAB staining

3.5. Microstructural study

The microstructural study by Cryo-SEM of the persimmon flesh samples submitted to different CO_2 exposure times is presented in Fig. 4. The observation of the cross-sections of astringent fruit (0h CO_2 treatment) demonstrated that the parenchyma cells of the mesocarp were irregularly shaped, but basically round. The interior of cells were filled with a soluble material, while the parenchyma was quite compact with small air-filled intercellular spaces (Fig. 4A, 4B).

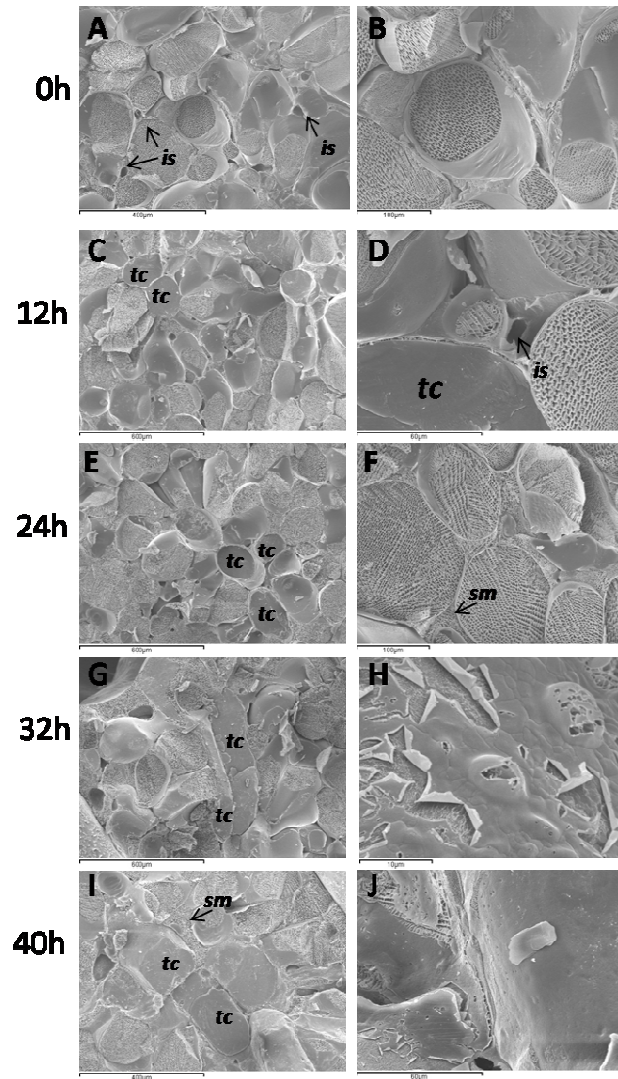


Fig. 4. Flesh structure of persimmon cv. Rojo Brillante analyzed by Cryo-SEM after being submitted to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 0, 12, 24, 32 and 40 h. *is*: intercellular space, *tc*: tannic cell, *sm*: soluble material.

No important differences between flesh samples of the 6h- and 12h- CO₂-treated fruit were observed. So only the micrographs of the fruit treated for 12 h are shown (Fig. 4C, 4D). The parenchyma structure had the same appearance as that of untreated fruit (0h-CO₂). However a major change was observed in some cells, which appeared to be filled with an insoluble material (like a compact mass), identified as tannic cells inside which tannins became insoluble due to CO₂ treatment.

The parenchyma structure of the 24-h CO₂-treated fruit was very similar to that seen after the 12h CO₂ treatment and, once again, with an insoluble material filling the vacuoles of tannic cells. In this case, while some intercellular spaces remained air-filled, others were filled with a soluble material (Fig. 4E, 4F).

The samples taken from the 32h- (Fig. 4G, 4H) and the 40h-treated (Fig. 4I, 4J) fruit showed progressive parenchyma structure degradation if compared to that of the 24h-treated fruit. Cells were more deformed, and the intercellular spaces were filled with a soluble material. In both samples, long-shaped tannic cells with an insoluble material inside were observed. Figure 4H and 4J illustrates the degraded aspect of cell membranes.

After the storage period, we selected sound astringent fruit (0h CO₂ hand-packed fruit), pinkish-bruised fruit (0h CO₂ mechanically packed fruit), sound non astringent fruit (24h- CO₂ hand-packed fruit) and browned fruit (24h CO₂ mechanically packed fruit) to study the microstructural changes associated with the appearance of flesh disorders. Cryo-SEM micrographies are provided in Fig. 5A. Sound astringent fruit (Fig. 5A1) and sound non astringent fruit (Fig. 5A3) showed a very similar parenchyma structure to that observed before storage; that is, cells with a defined shape and membranes of considerable integrity, with some intercellular spaces filled with air and others occupied by a soluble material in the case of non astringent fruit.

The observation made of the samples from the pinkish flesh areas (Fig. 5A2) revealed a much degraded parenchyma whose structure differed from that observed in sound astringent flesh (Fig. 5A1). A pinkish disorder was observed like a parenchyma in which many cells were void of their initial content, while the intercellular spaces were filled completely with a somewhat soluble, but mainly insoluble material.

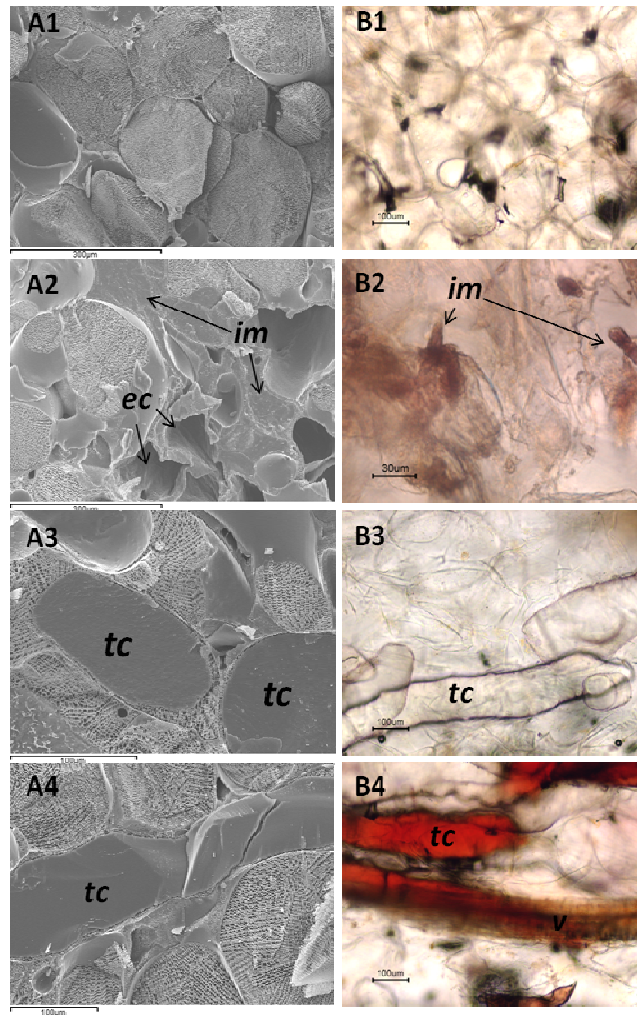


Fig. 5. Flesh structures of persimmon cv. Rojo Brillante analyzed by Cryo-SEM (A) and optical microscopy (B) after 15 days of storage. Figures A1 and B1 show sound flesh of astringent fruit (0h-CO₂ treatment plus hand packing). Figures A2 and B2 illustrate pinkish-bruised flesh of astringent fruit (0h-CO₂ treatment plus mechanical packing). Figures A3 and B3 show sound flesh of non astringent fruit (24h-CO₂ treatment plus hand packing). Figures A4 and B4 depict flesh browning of non astringent fruit (24h-CO₂ treatment plus mechanical packing). *im*: insoluble material, *ec*: empty cells, *tc*: tannic cell, *v*: vessel.

The microstructure studies of browned flesh areas (Fig. 5A4) showed quite a degraded parenchyma. It must be stressed that these areas were characterized by the presence of long-shaped cells with an insoluble material inside; these cells were identified as tannic cells in which tannins precipitated after the deastringency treatment.

We combined the Cryo-SEM studies with optical microscopy observations of the fruit since the flesh disorders under study were manifested as changes in flesh color (Figs. 5B). A comparison of pinkish-bruised flesh (Fig. 5B2) with sound astringent fruit flesh (Fig. 5B1) revealed that the pinkish-bruising disorder was associated with cellular disruption and the presence of a pink insoluble material outside cells, which was not found in the sound areas.

When comparing the sound (Fig. 5B3) and browned flesh of non astringent fruit (Fig. 5B4), the browning disorder was associated with the presence of long-shaped red-brown cells, which were identified as tannic cells. In the sound areas of non astringent fruit, tannic cells resembled long-shaped colorless cells.

3.6. Chromatographic study of tannins

An extraction of persimmon tannins from flesh samples with an ethanol/water solution, followed by centrifugation, led to the separation of soluble tannins, which were extracted and remained in the supernatant, from insoluble tannins, which remained in the insoluble fraction after extraction (pellet). The acid depolymerization of tannins in the presence of a nucleophilic reagent converted the flavon-3-ols extension units of one chain into the corresponding thioeteres. In this study, thiolytic degradation was applied independently of the supernatant and flesh debris after the ethanolic extraction of tannins from non damaged astringent fruit (0h-CO₂ treatment plus hand packing), from browned fruit (24h-CO₂ treatment plus mechanical packing) and from sound non astringent fruit (24h-CO₂ treatment plus hand packing) after storage at 1°C.

When the tannins from astringent fruit were analyzed, a high percentage of total tannins was detected in the soluble fraction, as expected (Supplementary Fig. S3A), while only a small percentage was present in the insoluble form (Supplementary Fig. 3A). The study of the units forming soluble tannins revealed epigallocatechin-gallate (EGCg) and epigallocatechin (EGC) as the

flavan-3-ol units present in the highest proportion (9% and 4% dw, respectively), while the proportion of epicatechin (EC) and epicatechin-gallate (ECg) was much lower (<2% dw).

An analysis of the samples taken from the sound areas of the 24h CO₂-treated fruit showed that most tannins were in the insoluble flesh fraction, indicating that a tannins insolubilization process had occurred (Supplementary Fig. 3B). Once again, EGCg and EGC were present at much higher levels than EC and ECg.

A comparison of the tannins-forming units afforded by the thiol degradation of browned samples (fruit submitted to the 24h CO₂ treatment plus mechanical packing) with those afforded from sound non astringent flesh (fruit submitted to the 24h CO₂ treatment plus hand packing) revealed that these changes in tannins were associated with the browning manifestation (Supplementary Figs. S4 and 6). The thiol degradation of the soluble fraction (supernatant) of the samples taken from browned fruit revealed a slightly lower level of the EGCg units forming soluble tannins as compared to the level of this flavan-3-ol unit obtained from the soluble tannins of sound fruit (Supplementary Fig. S4).

When the thiol degradation was applied to the insoluble fraction (pellet) of the samples from browned fruit, a broad hump appeared on the HPLC baseline, which was not seen in the chromatographic profile of the insoluble tannins from sound fruit (Fig. 6). In order to investigate if the changes associated with the persimmon browning observed in this study are due to an oxidation process, a controlled oxidation with KO₂ was carried out on the samples from non stringent sound fruit. The thiol degradation of controlled oxidized samples revealed fewer EGCg units and slightly less EGC units forming soluble tannins when compared to the native sound non astringent fruit samples (Supplementary Fig. S4). Moreover, the analysis of the insoluble tannins after thiol degradation revealed that a broad hump appeared on the HPLC baseline (Fig. 6). Therefore the changes in tannins associated with flesh browning followed the same pattern as those observed after performing a controlled oxidation of tannins.

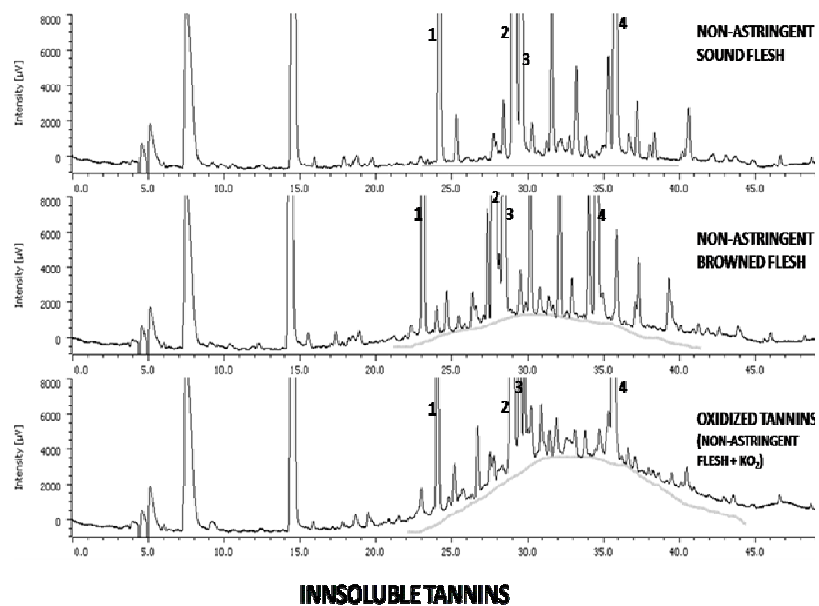


Fig. 6. Chromatogram (HPLC-DAD) profile of the thiol-degradation of insoluble tannins (remained in fleshy debris after ethanolic extraction) of the persimmon fruit subjected to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 24 h and then hand packed (non astringent sound fruit) or mechanically packed (non astringent browned fruit) or to controlled non enzymatic oxidation (oxidized tannins: non astringent fruit + KO₂).

4. Discussion

4.1. Tannins are implied in mechanical-induced flesh disorders

This work initially focused on studying the flesh browning disorder of the ‘Rojo Brillante’ persimmon. Nevertheless while this research line was underway, another flesh disorder was identified, which we named “pinkish-bruising”. Here, we attempt to collect data from the physiological, microstructural and chromatographic studies obtained in this paper, together with previous results from our laboratory, to help gain a better understanding of the flesh browning process in persimmon.

Exposure of fruit to 95% CO₂ for 24 h at 20°C is considered the standard treatment for ‘Rojo Brillante’ since it completely removes astringency (Salvador et al., 2007). Accordingly, the physiological study results reveal that from 0 to 24 h, the longer the CO₂ exposure, the lower the soluble tannins content. However, no differences were found in the soluble tannins content among the fruit overexposed to CO₂ (32h and 40h CO₂) as compared to the 24h-treated fruit. CO₂ treatments which last longer than 24 h gave soluble tannins values of 0.03%, which are habitual values in non astringent ‘Rojo Brillante’ persimmon (Besada et al., 2010b). The effectiveness of CO₂ treatment to remove astringency lies in the fact that it triggers anaerobic respiration in fruit, which gives rise to acetaldehyde accumulation, and then to a reaction between this acetaldehyde and soluble tannins, which are responsible for the astringency results in tannins becoming insoluble (Matsuo & Ito, 1982; Matsuo et al., 1991). In parallel to tannins insolubilization, the acetaldehyde concentration increased for the 0-24 h treatment, but its accumulation was slightly higher in the 32h- and 40h-treated fruit than in the 24h-treated fruit. Total antioxidant capacity was seen to parallel the content of soluble tannins, which are known to possess a high antioxidant capacity (Gulcin, Oktay, Kirecci & Kufrevioglu, 2003; Minussi et al., 2003). We previously reported a drastic drop in the total antioxidant capacity of ‘Rojo Brillante’ persimmons after being submitted to the standard deastringency process (Besada et al., 2012).

The evaluation of the flesh disorders manifestation revealed that, irrespectively of the number of hours that the fruit were exposed to CO₂, hand-packed fruit (not mechanically impacted) did not display flesh disorders, while mechanically packed fruit showed browning, pinkish-bruising, or both disorders together, depending on the level of astringency. Therefore, mechanical impacts are herein confirmed as the factor that triggers not only browning, but also

pinkish-bruising development. In previous studies, we found that non astringent fruit, submitted to CO₂ treatment under standard conditions (24h CO₂), were seen to be strongly susceptible to displaying browning after packing operations, while this disorder was not exhibited in astringent fruit (Besada et al., 2012). In the present work, the incidence of the two flesh disorders under study correlated well with the level of soluble tannins present in the fruit at the time of mechanical damage; pinkish-bruising correlated positively with soluble tannins content, while browning correlated inversely. Therefore, the browning manifestation is associated with the level of insoluble tannins. Moreover, the manifestation of both disorders correlated directly or inversely with the level of acetaldehyde and the antioxidant capacity of fruit upon packing.

The good correlation found between flesh disorders manifestation and tannin content, acetaldehyde concentration and the antioxidant capacity of fruit after CO₂ treatments suggests that these three factors are implied in the mechanical damage-induced disorders process. However, other factors associated with CO₂ treatment must be involved; since in the fruit exposed to CO₂ for periods longer than 24 h, the browning incidence was enhanced in prolonged treatments although the level of soluble tannins and antioxidant capacity did not indicate a more marked decrease

4.2. CO₂-treatment and mechanical damages induce ROS accumulation

The ROS study results reveal significant O₂⁻ accumulation with hours of exposure to CO₂. However, no H₂O₂ accumulation was observed. Excessive ROS generation, i.e., under oxidative stress, is an integral part of many stress situations, including hypoxia (Blokina et al., 2003). ROS generation mechanisms in biological systems are represented by both non enzymatic and enzymatic reactions. Among the enzymatic sources of ROS, xanthine oxidase (XOD) is a key enzyme that is responsible for initial dioxygen activation. As electron donors, xanthine oxidase can use xanthine, hypoxanthine or acetaldehyde (Bolwell & Wojtaszek, 1997). Acetaldehyde represents a possible source of hypoxia-stimulated ROS production. In this sense, Gong & Mattheis (2003) studied the browning of 'Braeburn' apple under low oxygen and anaerobic conditions to report that addition of exogenous acetaldehyde before fruit storage resulted in enhanced XOD and NADH oxidase activity, and in lesser SOD activity which, in turn, results in superoxide accumulation. In the present study, the greater AcH accumulation in fruit as longer hours of exposure

to CO₂, i.e., the acetaldehyde concentration-time combination, could account for the greater accumulation of superoxide anion, as observed for a longer CO₂ treatment, since acetaldehyde is a substrate for XOD activity, which leads to superoxide (O₂⁻) generation.

At this point, we must bear in mind that although CO₂ treatment leads to O₂⁻ accumulation, the treatment itself does not result in flesh disorders, and mechanical damage is necessary to trigger browning and pinkish-bruising development in persimmon. It must be considered that O₂⁻, as a charged species, cannot penetrate biological membranes.

In this work, the *in vivo* detection of ROS in mechanically packed fruit displaying flesh disorders reveals that both browning (manifested in non astringent fruit) and pinkish-bruising (manifested in astringent fruit) are associated with enhanced O₂⁻ levels and with the appearance of detectable H₂O₂ levels. Peroxide levels are not present in those flesh areas which remain sound. Therefore in both astringent and non astringent fruit, mechanical impacts trigger the generation of O₂⁻ and H₂O₂. Moreover, mechanical damage has been related to oxidative burst in other fruit, such as apricots and pears (De Martino et al., 2006; Li et al., 2010).

4.3. Microstructural changes associated to flesh disorders

Furthermore, the combination of optical and Cryo-SEM studies reveals that browning and pinkish-bruising disorders are paralleled by microstructural changes. The browning manifestation, which inversely correlates with soluble tannins, and must, therefore, correlate positively with insoluble tannins, is associated with the presence of large-sized cells filled with an insoluble material, identified as tannic cells, and in which the insolubilization of tannins took place during CO₂ treatment. The microscopy optical observation demonstrates that these cells are intensely red-brown colored, while no colored cells were encountered sound flesh. Yang, Ruan, Wang and Li (2005) carried out a morphological characterization of tannic cells from different persimmon cultivars at harvest. They classified tannic cells by optical microscopy into six groups according to shape and into four groups according to surface performance. These authors reported that colorless and colored tannic cells can be distinguished and that these tend to be brown in naturally non astringent cultivars. In fact, it is not unusual that some non astringent cultivars show

naturally flesh browning which, to the naked eye, looks very similar to the mechanical induced-browning of the astringent ‘Rojo Brillante’ cultivar. In the above-cited work, the authors suggested that the color of tannin cells could be due to cellular oxidation. However, this was not the objective of their study and no evidence for this suggestion was provided.

4.4. Tannins oxidation process is behind the flesh disorders

In the present research work, the chromatographic study reveals that the changes in insoluble tannins associated with browning manifestation show the same pattern as those changes associated with controlled tannins oxidation by addition of ROS generator KO_2 , suggesting that the tannins oxidation process is behind the flesh browning manifestation. The changes noted in the present work (fewer flavan-3-ol units from soluble tannins and a broad hump appearing when applying thiol degradation to the insoluble fraction) have been previously related to the tannins oxidation process. Along these lines, Poncet-Legrand et al. (2010) reported a reduced area of flavan-3-ol unit peaks after tannins oxidation as compared to native peaks in apple fruit. Moreover after studying tea tannins, Tanaka, Matsuo and Kouno (2010) indicated the appearance of a broad hump on the HPLC baseline, which they described to be an uncharacterized polymer-like polyphenol product due to the oxidation of flavan-3-ols. Besides, it is known that one of the limitations of the thiolitic depolymerization of tannins is the non cleavage of new covalent bonds created by oxidation (Poncet-Legrand et al., 2010).

4.5. Suggested mechanism of flesh disorders

Tannins belong to the group of polyphenol compounds which possess an ideal structural chemistry for free radical scavenging activity. The antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and based on their ability to chelate transition metal ions. It was recently shown that phenolic compounds may be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama & Oniki, 1997).

Peroxide, which has been associated with the browning disorder, is the superoxide dismutation product. It is also a potentially hazardous compound, and an array of antioxidant enzymes, such as CAT and APX (Chen & Asada, 1989), and a variety of general peroxidases (POD) (Chang, Siegel & Sigel, 1984), catalyze the breakdown of H₂O₂. A wide range of hydrogen donors, including polyphenols, can be oxidized by POD, and the involvement of POD in enzymatic browning has been reported (Lopez-Serrano & Barcelo, 1997; Richard-Forgetand & Gauillard, 1997). Furthermore, we previously determined that POD enzyme activity increases with the intensity of the mechanical impact and browning intensity. Moreover hot water treatments, which reduce the browning manifestation, enhance CAT and APX activity, which are two enzymes implied in H₂O₂ scavenging (Khademi et al., 2012).

By taking in account all the changes associated with the browning manifestation, our results strongly suggest that this disorder results from the oxidative burst triggered by mechanical impacts in flesh tissue. Mechanical damage leads to the accumulation of H₂O₂ and O₂⁻, which must be greater the higher the level of O₂⁻ present in the fruit before being impacted. As a result of this redox imbalance, insoluble tannins must be oxidized in a process which implies ROS and enzymes such as POD. Due to this oxidation process, insoluble tannins change to a red-brown color, which results in flesh browning.

The microstructure changes associated with pinkish-bruising, which positively correlated with soluble tannins content at the time of the mechanical impact, differed, to some extent, from those observed for browning. Pinkish-bruising was seen to be an output of cellular content and the appearance of pink insoluble material in the intercellular spaces, which apparently result from an insolubilization process of the initial soluble material after leaving cells.

To date, no enzymatic or chromatographic studies have been carried out in our laboratory into the pinkish-bruising disorder. However, the informal sensory testing of pinkish flesh areas carried out throughout this study reveal that even in non CO₂-treated fruit, the flesh areas affected by the pinkish-bruising disorder are not astringent. Therefore, it is quite possible that the initial soluble tannins of astringent fruit become insoluble by an oxidation process, in which ROS must also be implicated. The oxidative insolubilization of tannins in mechanically impacted areas of astringent fruit would result in the pinkish-bruising manifestation.

One of the main differences between the pinkish-bruising and browning disorders is that the former is manifested in located areas, while browning tends to extend around the fruit. It must be stressed that fruit sensitivity to impact energy depends on viscoelastic tissue properties (Vergano, Testin & Newall, 1991). The insolubilization of tannins during CO₂ treatment is expected to confer rigidity to tannic cell contents. Besides, loss of membrane integrity is associated with CO₂ exposure. Thus, astringency removal, which results in tannic cells being filled with a rigid insoluble material, must enhance the sensitivity of fruit to damage while they roll on the packing line. Astringent fruit with cells filled with a soluble material must be able to absorb rolling damage, and it is likely that only strong energy impacts disrupt initial integrity. Given the good antioxidant capacity associated with soluble tannins, it is also expected that astringent fruit may rapidly return to a redox initial balance. However, non astringent fruit, submitted to CO₂ treatment, show not only a poor antioxidant capacity, but also a previously altered redox state due to O₂⁻ accumulation, which takes place during CO₂ treatment. Finally, the longer the CO₂ exposure the greater the O₂⁻ accumulation.

4.6. Concluding remarks

To summarize, although many questions still remain unanswered, e.g., if the browning and pinkish-bruising mechanism is only enzymatic or if it is a combination of enzymatic and non enzymatic reactions, this paper offers information about mechanical-induced disorders of persimmon fruit.

We report for the first time that CO₂ deastringency treatment applied to persimmon fruit results in superoxide anion accumulation. Moreover, the mechanical damage fruit are exposed to during packing operations also triggers oxidative stress. This imbalance in the redox state may lead to a tannins oxidation process, which results in the manifestation of flesh disorders depending on the level of soluble/insoluble tannins. Flesh browning is observed in fruit that have previously been subjected to deastringency treatment, while pinkish-bruising is manifested in fruit that remain astringent. This information may also prove a useful starting point to study flesh browning that is observed naturally in non astringent cultivars.

Acknowledgments

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Supplementary data

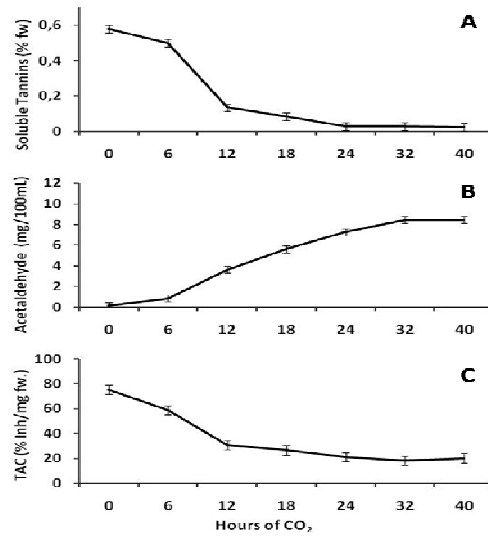


Fig. S1. Soluble tannin content (% fw) (A), acetaldehyde concentration (mg/100mL) (B) and total antioxidant capacity (% Inh/g) (C) of persimmon cv. Rojo Brillante submitted to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 0, 6, 12, 18, 24, 32 and 40 h. Vertical bars represent the LSD test (P>0.05)

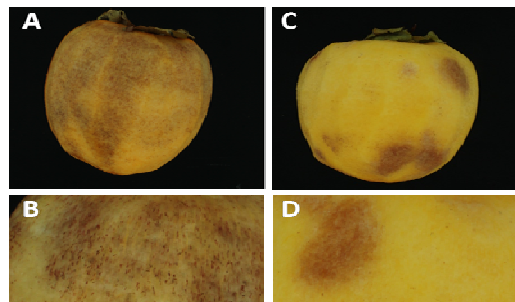


Fig. S2. Flesh browning (A, B) and pinkish-bruising (C, D) observed in persimmon cv. Rojo Brillante subjected to mechanical packing before storage. Flesh browning was observed in fruit submitted to the destringency treatment with CO₂. Pinkish-bruising was observed in astringent fruit not submitted to the destringency treatment.

Table S1. The Disorder Index (Pinkish-Bruising/ Browning) shown by persimmon cv. Rojo Brillante submitted to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 0, 6, 12, 18, 24, 32 or 40 hours plus mechanical packing and then stored for 15 days at 15°C (A) or 1°C (B).

	Pinkish (15°C)	Pinkish (1°C)	Browning (15°C)	Browning (1°C)
0h-CO ₂	0,46	0,68	0	0
6h-CO ₂	0,38	0,61	0	0
12h-CO ₂	0,25	0,46	0,03	0,1
18h-CO ₂	0,21	0,41	0,33	0,38
24h-CO ₂	0,1	0,28	0,61	0,71
32h-CO ₂	0	0	0,68	0,87
40h-CO ₂	0	0	0,71	0,94

Table S2. Correlation between soluble tannin content, concentration of acetaldehyde or total antioxidant capacity of persimmon cv. Rojo Brillante submitted to treatment with high concentration of CO₂ (95% CO₂, 20°C, 90% H.R.) for 0, 6, 12, 18, 24, 32 and 40 h and the Disorder Index (Pinkish-Bruising/ Browning) shown by fruit after being mechanically packed and stored for 15 days at 15°C or 1°C.

	Soluble Tannins (% fw)	Acetaldehyde (mg/100mL)	Antioxidant Cap (%Inh/ mg)
Pinkish (15°C)	$r= 0,92$	$r= -0,98$	$r= 0,83$
Pinkish (1°C)	$r= 0,83$	$r= -0,95$	$r= 0,73$
Browning (15°C)	$r= -0,81$	$r= 0,95$	$r= -0,65$
Browning (1°C)	$r= -0,81$	$r= 0,96$	$r= -0,67$

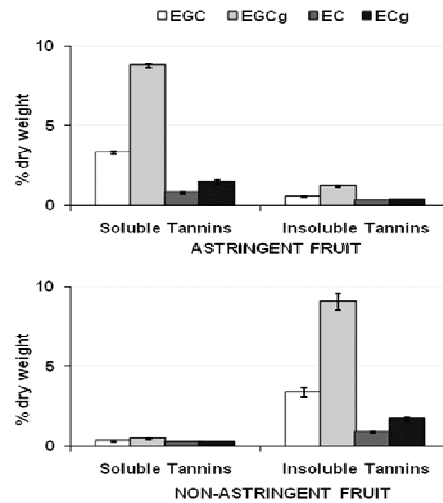


Fig. S3. Proportion (% of dry weight) of repeating subunits (flavan-3-ol) forming the soluble and insoluble tannins of astringent persimmon cv. Rojo Brillante (0h- CO₂) (A) and after being submitted to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% HR) for 24 h (non astringent fruit) (B). Vertical bars represent standard deviation.

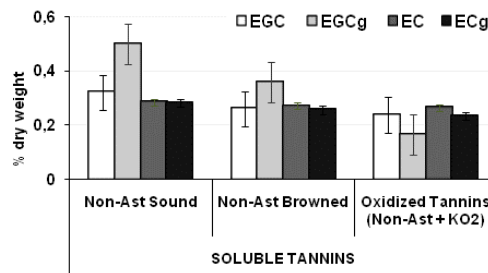


Fig. S4. Proportion (% of dry weight) of repeating subunits (flavan-3-ol) forming the soluble tannins of persimmon fruit subjected to treatment with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.) for 24 h and then hand packed (non astringent sound fruit), or mechanically packed (non astringent brownd fruit) or to controlled non enzymatic oxidation (oxidized tannins: non astringent fruit + KO₂). Vertical bars represent the LSD test (P>0.05)

CHAPTER II

Sensitivity of astringent and non-astringent persimmon cultivars to flesh disorders induced by mechanical damage

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Abstract

Recently, two types of flesh disorders (browning and pinkish-bruising) induced by mechanical damage during packing operations have been described in astringent persimmon 'Rojo Brillante'. Tannins, in their soluble and/or insoluble form, have been shown to be involved in the manifestation of both disorders. Persimmon cultivars can be classified into two types, astringent and non astringent cultivars, depending on the level of soluble tannins at harvest time. The influence of persimmon type in flesh disorder sensitivity has been not studied. The objective of this work was to evaluate the sensitivity to flesh disorders induced by mechanical damage in astringent cultivars (cv. Atago, cv. Giombo, cv. Aizumishirazu) and non-astringent cultivars (cv. Cal Fuyu, cv. Jiro, cv. O'goshō). Persimmon fruits submitted or not to deastringency treatment were mechanical-packaged in order to induce mechanical damage. Hand-packed fruit acted as control. Soluble tannin content, acetaldehyde and ethanol production, antioxidant capacity, astringency sensory evaluation and incidence of flesh browning and pinkish-bruising were evaluated. Our results showed that non-astringent cultivars exhibit low sensitivity to browning/pinkish-bruising by mechanical damage. In contrast, astringent cultivars showed browning and/or pinkish-bruising depending on the level of soluble tannins. The incidence of these disorders depends on the cultivar.

Keywords: Browning, Pinkish-bruising, Soluble tannins, Astringency, Deastringency treatment

INTRODUCTION

Persimmon cultivars can be classified into astringent and non-astringent depending on the level of soluble tannins at harvest time. Astringent cultivars, due to the high soluble tannin content, are usually submitted to the CO₂-treatment to remove astringency to be ready for consumption. It is based in the insolubilization of tannins by intermediation of the acetaldehyde generated during anaerobic respiration, which is triggered during exposition of fruit to high-CO₂ atmosphere (Matsuo et al., 1991).

In astringent cv. Rojo Brillante it has been recently described two types of flesh disorders depending on the level of insoluble/soluble tannins: flesh browning and pinkish-bruising. These alterations have been associated with tannins oxidation process due to oxidative stress triggered by both CO₂-destringency treatment and mechanical damage suffered by the fruit during packing operations (Novillo et al., 2014a). The level of astringency of the fruit at the moment of receiving a mechanical impact has been reported to be determinant for the incidence of both disorders. Fruit with high level of astringency shows high susceptibility to display pinkish-bruising after packing operations while non-astringent fruit (submitted to CO₂-treatment) shows high susceptibility to manifest browning (Besada et al., 2010).

This study aimed to investigate the influence of mechanical damage and CO₂-treatment on the sensitivity to flesh disorders in several astringent and non-astringent persimmon cultivars.

MATERIALS AND METHODS

Plant Material

In the present work has been explored the fruit sensitivity to flesh disorders of six persimmon cultivars: cv. Atago, cv. Giombo, cv. Aizumishishirazu-A (astringent cultivars) and cv. Cal Fuyu, cv. Jiro, cv. O'goshō (non astringent cultivars).

The persimmons of the different studied cultivars were harvested at commercial maturity stage in the mid-season (late November); from the

persimmon germplasm collection hosted by the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). The external color measured as color index (CI) ranged from 10 to 20 and firmness values ranged between 25-50 Newtons.

After harvest, the fruits were taken to the Instituto Valenciano de Investigaciones Agrarias (IVIA) and six lots of 15 fruits from each cultivar were performed. Three lots were submitted to destringency treatment (95% CO₂ at 20 °C and 90% R.H, during 24h) and the other three lots were not subjected to destringency process. One day after treatment, one lot of fruit submitted and fruit not submitted to CO₂-treatment was evaluated through measurements of soluble tannins (ST), total antioxidant capacity, acetaldehyde and ethanol production. Besides, astringency was evaluated by sensorial analysis.

In order to evaluate the influence of CO₂-treatment and mechanical damage on flesh disorders incidence (browning/pinkish-bruising), one of the two lots of fruit submitted/not submitted to CO₂-treatment was mechanical-packed. The other one was hand-packed acting as control. Finally, fruits were transferred to 1°C up to 20 days, period after which the flesh disorders were evaluated.

Destringency treatment was carried out in closed containers and these conditions were established by passing a stream of air containing 95% CO₂ through the containers.

Physiological Assessment

External color was determined using a Minolta colorimeter (Model CR-300 Ramsey, NY, USA). Hunter parameters 'L', 'a', 'b', were measured and results were expressed as color index: $CI=1000a/Lb$.

Flesh firmness was evaluated at harvest with a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA) using an 8-mm plunger. Results were expressed as load in Newtons (N) to break the flesh in each fruit on 180° sides after peel removal.

The total antioxidant capacity was determined according Novillo et al. (2014b). Values obtained were compared to the concentration-response curve of the standard Trolox solution expressed as micromoles of Trolox Equivalents (TE) per g of fresh weight.

Soluble tannins were evaluated using the Folin-Denis method (Taira, 1995), and results were expressed in percentage of fresh weight.

Acetaldehyde and ethanol concentration was measured from juice sample and analyzed by headspace gas chromatography as described by Salvador et al. (2004); results were expressed as mg/100mL.

The sensory evaluation of astringency was performed using a four-point scale was used, where 0 was no astringency and 3 was very high astringency. Samples were presented to members of the panel in trays labeled with random three-digit codes and served at room temperature ($25 \pm 1^\circ\text{C}$). The judges had to taste several segments of each sample in order to compensate, as far as possible, for the biological variation of the material. Milk was provided for palate rinsing between samples.

Flesh disorders were visually evaluated after peel removed. The severity of the flesh disorders was rated on a four point scale according to the intensity: 0-absence; 1-slight (less than 10% of fruit flesh surface was affected); 2-mild (more than 10% and less than 30% of fruit flesh surface was affected); 3-moderate (more than 30% and less than 60% of fruit flesh surface was affected) and 4-severe (more than 60% of fruit flesh surface was affected). In order to obtain a unique value that reflects both the incidence and severity of each disorder, the following disorder index was calculated: $\Sigma [(disorder\ severity) \times (number\ of\ fruit\ at\ each\ disorder\ severity)] / (total\ number\ of\ fruits)$.

The data were subjected to the analysis of variance, and multiple comparisons between means were determined by the least significant difference test ($P = 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD).

RESULTS AND DISCUSSION

In the present study, as expected, at harvest time astringent cultivars showed a high ST content ($>0.35\%$ FW) compared to the values of non-astringent cultivars ($<0.05\%$ FW) (Fig.1A). After deastringency treatment while the ST content of non-astringent cultivars remained unchanged, the astringent cultivars cv. Giombo and cv. Aizumishishirazu-A exhibited a drastic decrease

of ST to values around 0.03 % FW. The cultivar 'Atago' showed slightly higher values (0.07 % FW).

Sensory evaluation revealed that destringency treatment was effective on removing astringency in the astringent cultivars 'Giombo' and 'Aizumishishirazu-A', which were evaluated by the panelist as "no astringency" (sensory value of 0) (data not shown). However, cv. Atago was evaluated as "slightly astringent". It would be explained by the slightly higher values of ST observed in this cultivar after destringency treatment. It has been reported that soluble tannin content below 0.1% FW in persimmon fruit does not produce astringency sensation (Vidrih et al., 1994). However in some varieties of persimmon soluble tannin content as low as 0.06% FW can produce a detectable astringency (Besada et al., 2010).

Numerous reports have indicated that the decrease in soluble tannins observed during astringency removal treatment is related to the accumulation of acetaldehyde in the flesh (Matsuo and Ito, 1982; Besada et al., 2010). As above mentioned, soluble tannins responsible of astringency are polymerized by acetaldehyde produced under anaerobic conditions to form insoluble compounds, which are non-astringent (Matsuo and Ito 1982; Taira et al., 1997). In this research, all the studied cultivars showed values of acetaldehyde production below 0.6 mg/100mL at harvest time (Fig.1B). After applying the destringency treatment, the acetaldehyde production significantly increased in all cultivars, excluding cv. Atago. As expected, the increase in acetaldehyde concentration when fruit exposed to anaerobic conditions was parallel to a significant accumulation of ethanol. In the most of cultivars the ethanol production was not detected at harvest time, but after CO₂-treatment increased until values around 50 mg/100mL (data not shown).

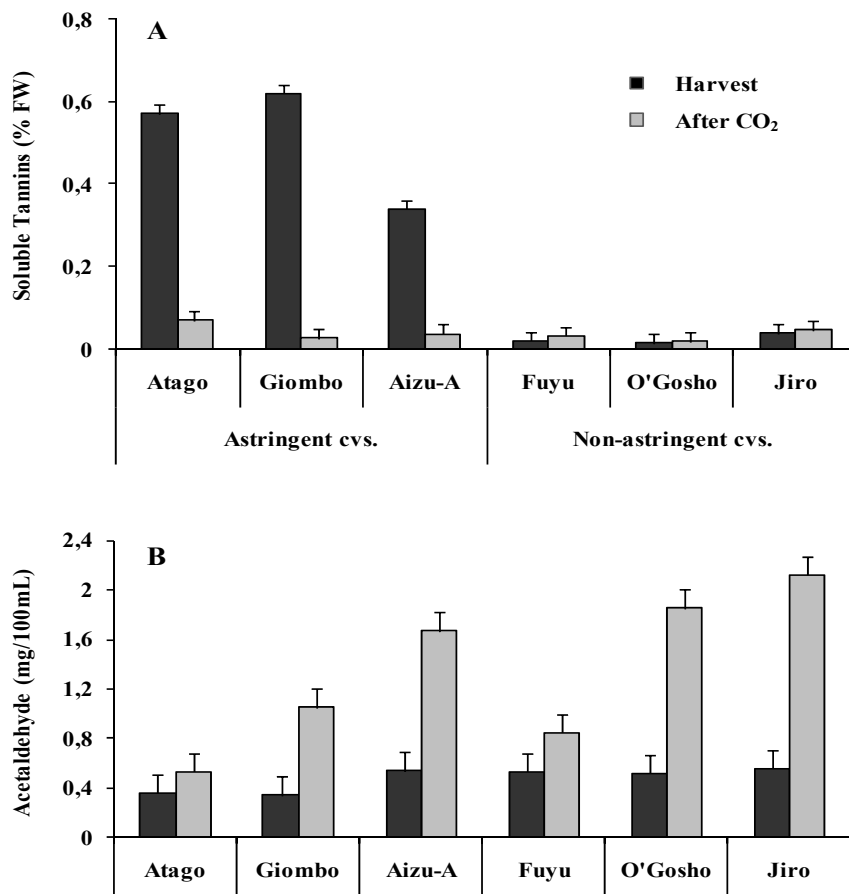


Fig. 1. Soluble tannins (A) (% FW) and acetaldehyde concentration (B) (mL/100mL) at harvest time and after CO₂ treatment from persimmon cultivars: cv. Atago, cv. Giombo, cv. Aizumishishirazu-A (astringent cultivars) and cv. Cal Fuyu, cv. O'gosho, cv. Jiro (non-astringent cultivars). Verticals bars represent LSD intervals (P< 0.05).

According to previous results, the total antioxidant capacity was closely related to the ST content. Soluble tannins are well-known for possessing radical scavenging activity (Gu et al., 2008). Thus at harvest in the astringent cultivars the total antioxidant capacity was much higher (values > 40 $\mu\text{mol TE/g FW}$) than in the non-astringency cultivars (values around 2 $\mu\text{mol TE/ g FW}$) (Fig.2).

Then, after destringency treatment while no changes in total antioxidant capacity were observed in non-astringent cultivars, in astringent cultivars the values dropped close to 2 $\mu\text{mol TE/g FW}$.

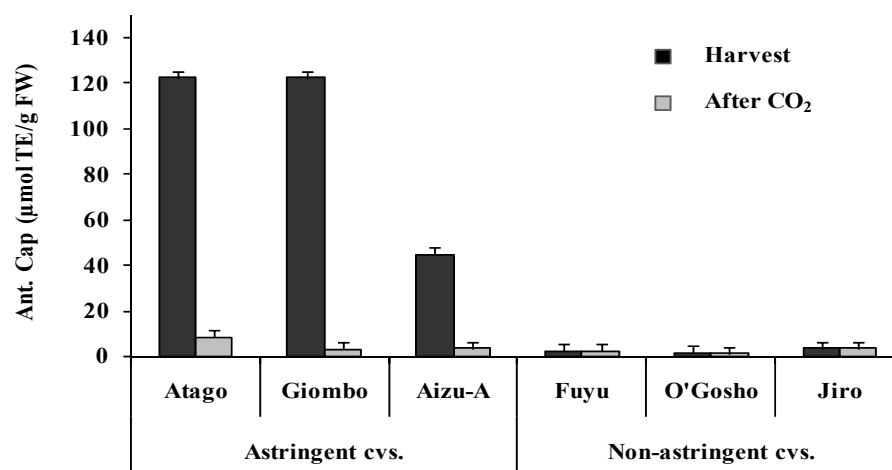


Fig. 2. Total antioxidant capacity ($\mu\text{mol TE/g FW}$) at harvest time and after CO_2 treatment from persimmon cultivars: cv. Atago, cv. Giombo, cv. Aizumishishirazu-A (astringent cultivars) and cv. Cal Fuyu, cv. O'goshu, cv. Jiro (non-astringent cultivars). Vertical bars represent LSD intervals ($P < 0.05$).

The evaluation of flesh disorders after cold storage revealed no incidence of browning or pinkish-bruising in hand-packed fruits from all astringent and non-astringent cultivars. Mechanical-packaged fruit from non-astringent cultivars also did not show these flesh disorders, but the mechanical damages were observed as uncolored-bruised areas in the flesh.

Nevertheless when fruit from the astringent cultivars was mechanical-packaged, browning and pinkish-bruising were detected (Table 1). Flesh browning was mainly observed in fruit subjected to destringency treatment with low level of ST and low antioxidant capacity, while pinkish-bruising was

manifested in fruit non-CO₂-treated with high ST content and high antioxidant capacity. These results are in accordance with that previously reported in the astringent cultivar ‘Rojo Brillante’ persimmon (Novillo et al., 2014b).

Table 1. Disorder index of browning/pinkish-bruising after mechanical-packing and cold-storage period of non-CO₂-treated or CO₂-treated fruit from astringent persimmon cultivars: cv. Atago, cv. Giombo, cv. Aizumishishirazu-A. Different letters in the same column indicate significant differences (95% LSD-test).

	Browning		Pinkish-Bruising	
	Non CO ₂ -treated	CO ₂ -treated	Non CO ₂ -treated	CO ₂ -treated
cv. Atago	0a	0.55a	0.91b	0.77b
cv. Giombo	0.4b	2.16c	1.68c	0a
cv. Aizu-A	0a	0.72b	0.70a	0a

The sensitivity of both flesh disorders depended on the cultivar. ‘Giombo’ was the cultivar with the highest pinkish-bruising index (1.68) in non-CO₂-treated fruit (astringent fruit) and the highest browning index (2.16) in fruit submitted to CO₂-treatment (non-astringent fruit) (Table 1 and Fig. 3). ‘Atago’ and ‘Aizumishishirazu-A’ showed a lower sensitivity to these disorders than cv. Giombo. It is noteworthy that in cv. Atago submitted to CO₂-treatment not only flesh browning but also pinkish-bruising was detected. This fact is explained by the level of ST observed in this cultivar after CO₂-treatment, when the fruit was evaluated as “slightly astringent”. Therefore the deastringency treatment was not completely effective and part of tannins have been insolubilized but there are still tannins in their soluble form.



Fig 3. cv. Giombo submitted to deastringency treatment with CO₂ plus hand-packing (sound flesh) (A) or mechanical-packing (flesh browning) (B) and then storage at 1°C up to 20 days.

CONCLUSSIONS

The results of the present study strongly suggest that sensitivity of persimmon to flesh disorders associated with mechanical damage is influenced by the persimmon type, astringent or non-astringent at harvest.

The non-astringent studied cultivars did not exhibit browning/pinkish-bruising even when the fruits were submitted to CO₂-treatment before being mechanical impacted, but manifested uncolored-bruised flesh areas associated with mechanical damage.

The astringent cultivars displayed browning and/or pinkish-bruising in fruit mechanical-packaged depending on the level of soluble/ insoluble tannins. Flesh browning was mainly observed in fruit with low level of soluble tannins (CO₂-treated), while pinkish-bruising was manifested in fruit with high soluble tannins content (non-CO₂-treated). Furthermore, the sensitivity to flesh disorders depended on the cultivar.

ACKNOWLEDGEMENTS

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Deastringency treatment with CO₂ induces oxidative stress in persimmon fruit

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Abstract

Given their condition of being astringent at harvest, persimmons cv. Rojo Brillante are regularly submitted to deastringency treatment based on exposing fruit to a high CO₂ concentration. The treatment conditions that ensure total astringency removal throughout the various maturity stages have been determined to be 95% CO₂, 20°C, 24 h. The aim of this study was to investigate changes in the redox state of persimmon associated with deastringency treatment. To that end, the level of reactive oxygen species (ROS) (O₂⁻ and H₂O₂), and the activity of the main ROS scavenging enzymes (CAT, POD, APX, and SOD), were determined at harvest and after deastringency in fruit in three different maturity stages. Our results showed that during 'Rojo Brillante' persimmon maturation, the level of O₂⁻ gradually incremented, while APX activity lowered. The deastringency treatment with CO₂ induced oxidative stress in fruit, observed as an over-accumulation of O₂⁻ and H₂O₂. As a response to ROS accumulation, the activity of the CAT, APX and SOD scavenging enzymes was up-regulated after deastringency treatment. The response of the POD enzyme was dependent on maturity stage, showing enhanced activity after CO₂ treatment only for the fruit in the most mature stage.

Keywords: Astringency, Tannins, Reactive Oxygen Species, Antioxidant system enzymes, Persimmon maturity, cv. Rojo Brillante

1. Introduction

Persimmon cv. Rojo Brillante belongs to the group of persimmon cultivars that are astringent at harvest given the high soluble tannins content in the flesh of this fruit. This means having to apply a postharvest deastringency treatment before fruit can be marketed. The application of this treatment at 95% CO₂ for 24 h at 20°C has been established as the optimal conditions to ensure the removal of 'Rojo Brillante' persimmon astringency throughout the season (October to December) (Salvador et al., 2007; Besada et al., 2010a). The effectiveness of CO₂ treatment to remove astringency is based on the insolubilization of tannins by the intermediation of the acetaldehyde generated during anaerobic respiration, which is triggered while fruit is exposed to a high CO₂ atmosphere (Matsuo et al., 1991).

Production of reactive oxygen species (ROS), O₂⁻, OH⁻ and H₂O₂, in plant cells is constitutive, but their level is often enhanced under environmental stress. Low-oxygen atmospheres have been reported to induce oxidative burst plants (Blokhina et al., 2003). The effective destruction of ROS requires the action of several ROS scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), to act concomitantly with non enzymatic antioxidants, such as ascorbic acid, glutathione, flavonoids, carotenoids, tocopherols, etc.

Several approaches have been employed to study the effect of CO₂ treatment on persimmon fruit. Many reports have addressed the changes in physiological parameters related to level of astringency (tannins content, acetaldehyde and sensory astringency) (Vidrih et al., 1994; Ahmed and Sobieh, 2007; Besada et al., 2010a) and to the maturity process (firmness, total soluble solids, respiration rate, ethylene rate, vitamin C or sugars) (Salvador et al., 2007; Del Bubba et al., 2009). Moreover, the effect of this treatment on the flesh structure (Salvador et al., 2007) and volatile active compounds (Besada et al., 2013) has also been described.

As in plants, it is expected that exposure of fruit to a low-oxygen atmosphere as extreme as 95% CO₂ will involve environmental stress and will lead to changes in the redox state of the fruit. It is known that as a result of astringency removal, and therefore of the insolubilization of tannins, the total antioxidant capacity of fruit is severely affected by CO₂ treatment (Besada et al., 2012). However no information is available about how CO₂ treatment can affect the level of ROS and the antioxidant enzymes system.

Furthermore, fruit ripening can be considered a stressful process when oxidation progressively increases, that is, as a functionally modified form of senescence (Rogiers et al., 1998). Oxidative stress associated with fruit ripening has been reported in species such as tomato (Mondal et al., 2004), mango (Singh and Dwivedi, 2008), peach (Camejo et al., 2010) and papaya fruit (Couto et al., 2012). However, changes in the pattern of anti-oxidative enzymes activity during this process depend on the species, and even on the cultivar. As example of this, CAT activity has been shown to increase during the ripening of tomato (Mondal et al., 2004) and guava (Mondal et al., 2009), while it decreased for saskatoon (Rogier et al., 1998), and while peaks in activity during ripening in papaya fruit have been observed (Couto et al., 2012).

Despite the changes occurring in many physiological parameters associated with persimmon maturation having been extensively studied (Ramin and Tabatabaie, 2003; Salvador et al., 2007; Del Bubba et al., 2009), no studies have addressed the redox state of fruit during the maturity process.

On the other hand recent studies have detected the implication of oxidative stress on persimmon postharvest disorders such as chilling injury (Zhang et al., 2010) and flesh browning (Khademi et al., 2012), however, no information is available about how redox status of fruit may be affected by the commonly applied deastringency treatment.

The objective of this work was to study the effect of CO₂ deastringency treatment on the redox state of persimmon cv. Rojo Brillante. For this purpose, ROS (O₂⁻ and H₂O₂) levels, the activity of ROS scavenging enzymes (CAT, POD, APX and SOD) and total antioxidant capacity have been evaluated at harvest in fruit in three different maturity stages and after submitting fruit to the deastringency treatment.

2. Material and Methods

2.1. Plant material and experimental design

Persimmon (*Diospyros kaki* Thunb.) cv. Rojo Brillante fruit were harvested in l'Alcúdia (E Spain) in three different maturity stages. The maturity index used for harvesting was external color of fruit; Hunter parameters 'L', 'a', 'b', were measured and the results were expressed as a color index: CI=1000a/Lb

(Jimenez-Cuesta et al., 1981). External color of the fruit at harvest ranged from stage SI (orange-green) corresponding to Color Index (CI)= 0.82 to stage SIII (orange-red) with CI= 22.32.

After harvest, fruit were taken to the Instituto Valenciano de Investigaciones Agrarias (IVIA), where they were divided into two homogeneous lots of 20 fruits. One lot was directly analyzed after harvest, while the other lot was submitted to the postharvest deastringency treatment with CO₂.

The deastringency treatment was carried out under standard conditions (95% CO₂, 24h, 20°C, 90% RH) in closed containers. These conditions were established by passing a stream of air containing 95% CO₂ through the containers. After the deastringency treatment, fruit were kept at ambient temperature in an air atmosphere for 24 h.

Immediately after harvest and 1 day after the deastringency treatment, fruit were physiologically evaluated by taking measurements of firmness, color, soluble tannins content (ST), concentration of acetaldehyde (AcH) and ethanol (EtOH) and CO₂, and ethylene production. The redox state of the fruit was also studied by evaluating peroxide concentration (H₂O₂), superoxide concentration (O₂⁻) and the activity of the SOD, CAT, POD and APX enzymes.

2.2. Evaluation of physiological parameters

The skin color of 15 fruits was determined in a Minolta colorimeter (Model CR-300 Ramsey, NY, USA). Flesh firmness was evaluated with a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA) using an 8-mm plunger. The fruit firmness values were the average of 14 fruits. The results were expressed as load in Newtons (N) to break the flesh in each fruit after peel removal.

To determine soluble tannins, total antioxidant capacity and the concentrations of acetaldehyde and ethanol, lots of nine fruit were divided into three samples (3 replicates; 3 fruit per replicate) and were cut into four longitudinal parts. Two opposite parts were sliced and frozen at -20°C to determine soluble tannins. The other opposite parts of the fruit were placed in an electric juice extractor and the filtered juice was then used to determine the concentrations of acetaldehyde and ethanol. Soluble tannins were evaluated by the Folin-Denis method (Taira, 1995), as described by Arnal and Del Río

(2004). The results were expressed as a percentage of fresh weight (%fw). Total antioxidant capacity was determined as the antiradical activity of methanolic extracts. It was spectrophotometrically tested by measuring the drop in absorbance of the free radical DPPH, according to Brand-Williams et al. (1995). The drop in absorbance at 515 nm was monitored after 30 min when the reaction reached a plateau. The percentage of DPPH neutralized in the steady state (%Inhibition) was determined by the following equation: % Inh= ((Abs B – Abs E)/ Abs B)*100, where Abs B and Abs E are the absorbance of the blank (B) and the extract (E), respectively. The obtained DPPH inhibition percentages were referred to as mg of flesh. The concentrations of acetaldehyde and ethanol were measured in three replicates per juice sample and were analyzed by headspace gas chromatography as described by Salvador et al. (2004). The results were expressed as mg/100mL.

The ethylene and respiration rates were recorded in three replicates of two fruits, and were then analyzed by GC-FID and GC-TCD, respectively. For this purpose, 1 mL of the headspace sample was injected into a Perkin Elmer gas chromatograph, as described by Salvador et al. (2005). Ethylene production was expressed as $\mu\text{L C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$ and the respiration rate was denoted as $\text{mL CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$.

In order to evaluate the redox state of the fruit, immediately after firmness measurement a sample of flesh from the opposite side of four individual fruit was peeled and cut into small pieces and frozen with liquid N_2 to be ground and kept at -80°C until the H_2O_2 , O_2^- and enzymatic analyses.

2.3. H_2O_2 and O_2^- content

ROS content was determined over frozen tissue from four individual fruit. Hydrogen peroxide levels were determined according to Velikova et al. (2000) with some modifications. First, 1 g of flesh tissue was homogenized in an ice bath with 2 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12000 rpm for 20 min and 0.5 ml of the supernatant was added to 0.5 ml of 10mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M KI. Supernatant absorbance was measured at 390 nm. Hydrogen peroxide content was determined using a standard curve and was expressed as $\text{nmol g}^{-1} \text{ fw}$.

O_2^- content was estimated according to the method of Elstner (1976) with a slight modification. First, 1 g of flesh tissue was homogenized with 1.5 ml of 50 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 12000 rpm

for 20 min at 4°C. A reaction mixture consisting of 1 ml of 1 mM hydroxyammonium chloride and 1 ml of crude extract (supernatant) was incubated at 25°C for 1 h. Color was developed by the addition of 1 ml of 17 mM sulfanilic acid (in glacial acetic acid/water, 3:1 v/v) and 1 ml of 7mM 1-naphthylamine (in glacial acetic acid/water, 3:1 v/v) for 20 min at 25°C. The specific absorption at 530 nm was determined. Sodium nitrite was used as a standard solution to calculate O_2^- content.

2.4. Measuring enzyme activity

All the enzyme extraction procedures were conducted at 4°C over frozen tissue from four individual fruit. For CAT and POD, 2 g of frozen tissue were homogenized with 10 ml of 50mM sodium phosphate buffer (pH 7) containing 1mM ethylene diamine tetraacetic acid (EDTA), 2mM dithiothreitol (DTT) and 1% (w/v) polyvinyl-pyrrolidone (PVPP). APX was extracted in the same buffer with the addition of 0.5 mM of ascorbic acid.

For SOD extraction, 100 mg of frozen tissue were homogenized in 100 μ L cold 50 mM of K-phosphate buffer (pH 7) containing 5 mM of ascorbic acid, 1 mM EDTA, 1% (w/v) PVPP and 0.1% (v/v) Triton X-100. Homogenates were centrifuged at 12000 rpm for 25 min at 4°C. Supernatants were used for the enzymes assays.

CAT activity was determined according to the method of Chance and Maehly (1955) with some modifications. This involved monitoring the disappearance of H_2O_2 by recording the drop in absorbance at 240 nm of a reaction mixture containing 50 mM of sodium phosphate buffer (pH 7), 90 mM of H_2O_2 and 0.5 ml of CAT extract. The molar extinction coefficient of H_2O_2 at 240 nm was taken as $40\text{mM}^{-1}\text{cm}^{-1}$ (Duan et al., 2011). One unit of CAT activity was defined as the amount of enzyme that decomposed 1 μ mol of H_2O_2 min^{-1} .

The APX activity measurement was adapted from Zhang et al. (2009) with some modifications. Activity was assayed in a mixture containing 2 ml of sodium phosphate buffer (50 mM, pH 7), 1 mM of ascorbic acid and 0.5 ml of H_2O_2 (4mM). The reaction was initiated by the addition of 0.5 ml of enzyme extract. APX activity was determined by monitoring the drop in absorbance at 290 nm as ascorbate was oxidized. A molar extinction coefficient of $2.8\text{mM}^{-1}\text{cm}^{-1}$ (Duan et al., 2011) was used to calculate activity. One APX unit was defined as the amount of enzyme that caused a decrease in OD_{290} per min under

the assay conditions, and enzyme activity was expressed in units per gram of fw per minute.

POD activity was determined by measuring the increase in absorption at 470 nm according to Zhang et al. (2009) with some modifications. The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7), 90 mM of H₂O₂ and 2% guaiacol. The reaction was initiated by the addition of 0.3 ml of POD extract. A molar extinction coefficient of 26.6mM⁻¹ cm⁻¹ (Duan et al., 2011) was used to calculate activity. One POD unit was defined as the amount of enzyme that caused an increase in OD₄₇₀ per min under the assay conditions, and enzyme activity was expressed in units per gram of fw per minute.

SOD activity was performed by native polyacrylamide gel electrophoresis (PAGE) following the method described by Laemmli (1970). Enzyme activity staining was performed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971) with minor modifications. Briefly, the gel was first soaked in 25 ml of 1.23mM NBT for 15 min, was quickly washed in distilled water and was then soaked in the dark in 30 ml of 100 mM of potassium phosphate buffer (pH 7.0) containing 28 mM of 1,2-Bis(dimethylamino)ethane (TEMED) and 2.8×10^{-2} mM of riboflavin for another 15-minute period. After a brief wash in distilled water, the gel was illuminated on a light box for 20 min. The enzyme solution was subjected to native PAGE with 10% polyacrylamide gel and 10% glycerol (w/v), and the separation was performed at 120 V for 2:30 h.

2.5. Statistical analysis

Data were subjected to the analysis of variance, and multiple comparisons between means were made by the least significant difference test (P = 0.05) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD).

3. Results

Table 1 presents the main parameters defining the maturity stage of fruit at harvest. We focused on fruit color, firmness, the parameters relating to fruit astringency (soluble tannins, acetaldehyde and ethanol), ethylene and CO₂ rate and antioxidant capacity. The fruit in maturity stage SI showed a color index of 0.82 corresponding to orange-green tones; that is, fruit with a mainly orange-colored peel, but with some areas that were still green. The fruit in maturity stage SII showed homogeneous orange tones (CI= 6.5), while the stage SIII ones had acquired orange-red coloration (CI=22). In parallel to the increase in color, fruit exhibited a gradual softening from 52N in stage SI to 26.6N in SIII; the greatest loss of firmness was observed from stage SII to SIII, which coincided with the most intensive advance in color. After the deastringency treatment, the mean color values were higher than those at harvest, while the firmness values were lower, although these differences were not statistically significant.

As the maturity process progressed, the soluble tannins content lowered from 0.78 %fw in maturity stage SI to 0.63 %fw in SIII. A slight decrease in total antioxidant capacity was observed from 79% to 75% Inh/mg, which was probably associated with the decline in soluble tannins since a positive correlation ($r=0.97$) between soluble tannins and antioxidant capacity was observed at harvest. Both parameters were markedly affected by the deastringency treatment; in the three maturity stages, the soluble tannins content drastically dropped to 0.03%fw, while the total antioxidant capacity values went below 30%Inh/mg.

Throughout maturation, the concentrations of acetaldehyde and ethanol remained constant, and both volatiles presented a concentration lower than 0.25 mg/100mL. A sharp rise in the concentration of volatiles took place after deastringency treatment; acetaldehyde reached values of 2.5-3mg/100mL, while ethanol rose to values of 45mg/100mL (in stages SI and SII) and of 18mg/100mL in stage III.

In this study, the fruit in stage SI showed the highest ethylene production at harvest, 2.50 nmol kg⁻¹ h⁻¹. From which point however, it declined to 1.66 nmol kg⁻¹ h⁻¹ in stage SII and to 0 in SIII. CO₂ production gave values of 0.29 nmol kg⁻¹ h⁻¹ and 0.27 nmol kg⁻¹ h⁻¹ in stage SI and stage SII, respectively, to then diminish to 0.21 nmol kg⁻¹ h⁻¹ in stage III. When fruit was submitted to deastringency treatment, ethylene production increased by about 2-fold in the

fruit in stages SI and SII in comparison to the levels at harvest; no changes were observed in the stage SIII fruit. CO₂ production enhanced slightly after deastringency in the fruit at stages SI and SIII. However it notably increased in the stage SII fruit.

Table 1. Values of color, firmness, soluble tannin content (ST), total antioxidant activity (TAA), acetaldehyde production (AcH), ethanol (EtOH), CO₂ and ethylene (C₂H₄) production in persimmon cv. Rojo Brillante in three harvest maturity stages and after being submitted to deastringency treatment (DA) with a high CO₂ concentration (95% CO₂, 20°C, 90% HR). For each maturity stage, values with different lower case letters are significantly different at $P < 0.05$. Different capital letters in each parameter indicates statistical differences at harvest ($P < 0.05$).

	Stage I		Stage II		Stage III	
	Harvest	After DA	Harvest	After DA	Harvest	After DA
Color Index (1000a/Lb)	0.82a ^A	1.35a	6.54a ^B	8.21a	22.34a ^C	24.12a
Firmness (N)	52.05a ^B	46.15a	48.11a ^B	41.68a	26.65a ^A	22.70a
ST (%fw)	0.78b ^B	0.04a	0.71b ^{AB}	0.04a	0.63b ^A	0.04a
TAA (% inh DPPH/mg)	79.18b ^C	22.36a	77.13b ^B	24.24a	75.40b ^A	15.07a
AcH (mg/100mL)	0.19a ^B	3.18b	0.18a ^{AB}	3.06b	0.17a ^A	2.55b
EtOH (mg/100mL)	0.22a ^C	45.85b	0.12a ^B	44.33b	0.09a ^A	18.70b
CO ₂ (mmol kg ⁻¹ h ⁻¹)	0.29a ^B	0.35b	0.27a ^B	0.70b	0.21a ^A	0.29b
C ₂ H ₄ (nmol kg ⁻¹ h ⁻¹)	2.50a ^C	5.42b	1.66a ^B	4.17b	0.00a ^A	0.00a

As far as ROS (O₂⁻ and H₂O₂) is concerned, the level of O₂⁻ detected in the stage SI fruit, 0.4 nmol g⁻¹, gradually increased as the maturation process continued and achieved values of 1 nmol g⁻¹ in stage SIII (Fig. 1A). Meanwhile, the H₂O₂ level at harvest exhibited values close to 0, irrespectively of the maturity stage (Fig. 1B). Deastringency treatment brought about a marked increase in the O₂⁻ level in all the maturity stages, with values of 2.5 nmol g⁻¹ being reached, regardless of the initial O₂⁻ level at harvest (Fig. 1A). Besides, major H₂O₂ accumulation was also detected after CO₂ treatment; the fruit stage

SI and SII obtained H_2O_2 values close to 30 nmol g^{-1} , while the maximum levels after deastringency, 50 nmol g^{-1} , were seen in stage SIII (Fig. 1B).

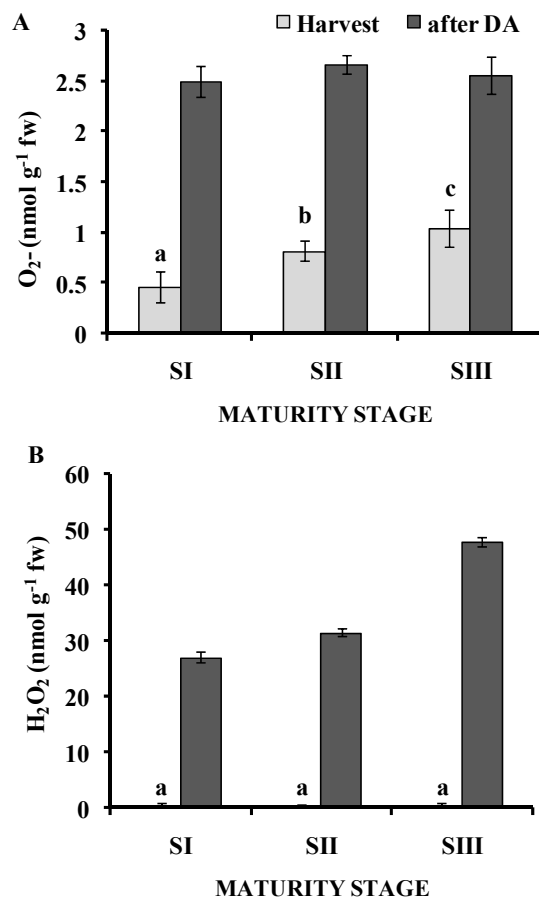


Fig. 1. Content of superoxide anion (O_2^-) (A) and peroxide (H_2O_2) (B) in persimmon cv. Rojo Brillante in three harvest maturity stages and after being submitted to deastringency treatment (DA) with a high CO_2 concentration (95% CO_2 , 20°C , 90% HR). For each maturity stage, vertical bars (LSD intervals) compare the values at harvest and after DA ($P < 0.05$). Different letters indicate statistical differences at harvest ($P < 0.05$)

By considering both the data from fruit at harvest and after the CO₂ treatment together, high inverse correlations were observed between the O₂⁻ level and soluble tannins ($r=-0.99$) and the O₂⁻ level and total antioxidant capacity ($r=-0.96$). No significant correlation was found between the fruit H₂O₂ level and the soluble tannins neither the total antioxidant capacity.

Regarding enzymes activity, the CAT enzyme values remained relatively constant, at around 1.5 U g⁻¹, in the three maturity stages (Fig. 2A). Enzyme activity was enhanced after CO₂ treatment, especially in the fruit in stages SI and SIII, when CAT activity increased by 2-fold as compared to the values obtained at harvest.

APX activity gradually declined with maturation; APX activity was 550 U g⁻¹ in the stage SI fruit, but then decreased to values of 124 U g⁻¹ in stage SIII (Fig. 2B). A major increment was observed in APX activity following deastringency treatment as compared to the levels at harvest. The fruit from stage SI showed maximum APX activity values after treatment, 1100 U g⁻¹, while fruit activity in stage SII and stage SIII was 750 and 600 U g⁻¹, respectively.

In this study, POD activity at harvest was of 3 U g⁻¹ in the fruit at all the maturity stages (Fig. 2C). Deastringency treatment did not affect the activity of this enzyme when it was applied to the stage SI and SII fruit. However, this treatment brought about POD activity values that were 2-fold higher than those at harvest when applied to the stage SIII fruit. A positive correlation was detected between POD activity and the H₂O₂ level after treatment ($r=0.99$).

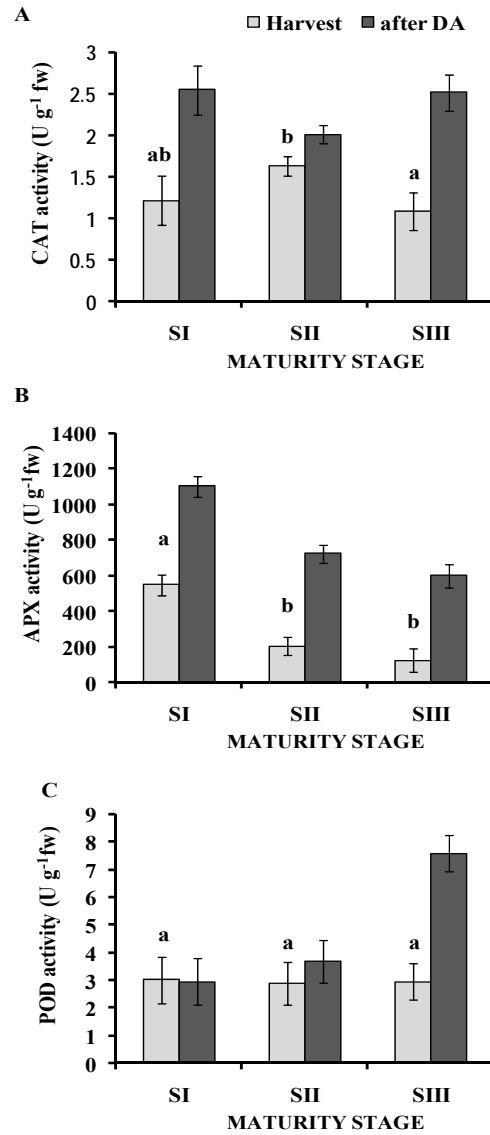


Fig. 2. Changes in the activity of catalase (CAT) (A), ascorbate peroxidase (APX) (B) and peroxidase (POD) (C) in persimmon cv. Rojo Brillante in three harvest maturity stages and after being submitted to deastringency (DA) with a high CO₂ concentration (95% CO₂, 20°C, 90% HR). For each maturity stage, vertical bars (LSD intervals) compare the values at harvest and after DA ($P < 0.05$). Different letters indicate statistical differences at harvest ($P < 0.05$)

The SOD enzyme was determined by native page (Fig. 3). This assay revealed the presence of one SOD isoenzyme at harvest, SOD 1, which became more active as the maturation process advanced. Deastringency treatment led to major changes in SOD; after treating fruit at the three tested maturity stages, isoenzyme SOD 1 was clearly seen to be up-regulated. Moreover, a new isoenzyme, SOD 2, appeared which was not detected at harvest.

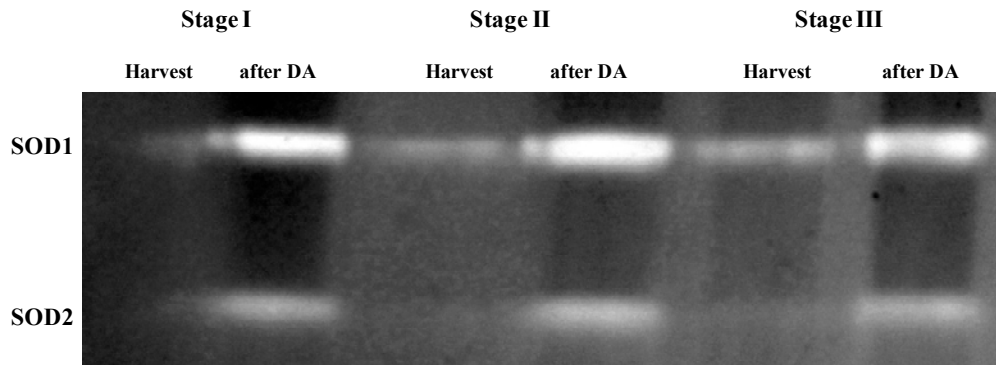


Fig. 3. Native-PAGE of superoxide dismutase (SOD) in persimmon cv. Rojo Brillante in three harvest maturity stages and after being submitted to deastringency treatment (DA) with a high CO₂ concentration (95% CO₂, 20°C, 90% H.R.). In each well, an equal concentration of protein extract was loaded (100µg/well).

4. Discussion

In this study, external fruit color at harvest was used to define three maturity stages (SI-SIII). Stage SI is not accepted for commercialization given the presence of some green-toned areas on fruit skins. However, stage SII and stage SIII are maturity stages that are usually marketed at the beginning and the end of the season, respectively. As expected (Salvador et al., 2007), advance in fruit color was accompanied by a progressive loss of firmness.

Persimmon fruit is a climateric fruit; although fruit produces low levels of ethylene, persimmons are very sensitive to external ethylene (Besada et al., 2010b). The climateric peak of 'Rojo Brillante' has been previously reported in the stage when fruit achieve homogeneous oranges tones (Salvador et al., 2007). Therefore in this study, values of $2.50 \text{ nmol kg}^{-1} \text{ h}^{-1}$ for stage SI and of $1.66 \text{ nmol kg}^{-1} \text{ h}^{-1}$ for stage SII must correspond to the ethylene production peak. In accordance with climateric behavior, maximum CO_2 production was also detected in both these maturity stages.

The persimmons belonging to the astringent group show a gradual decrease in soluble tannins during maturation. However even for advanced maturity states, soluble tannins remain at high levels and result in high fruit astringency (Suzuki et al., 2005). Soluble tannins are well-known for possessing radical scavenging activity (Gu et al., 2008). Accordingly, a positive correlation was observed between soluble tannins and total antioxidant capacity at harvest since both slightly lowered during fruit maturation.

The gradual increase in O_2^- as maturation advances suggests that persimmon ripening is accompanied by changes in the redox state of fruit, which has been previously reported in fruits such as guava (Mondal et al., 2009) or tomato (Mondal et al., 2004). Despite the high antioxidant capacity that soluble tannins give fruit, even in advanced maturity stages, it must be noted that superoxide is a charged molecule that cannot cross biological membranes, while tannins have been observed to accumulate in the vacuole of specific tannin cells. The subcellular compartmentalization of defense mechanisms is, therefore, crucial for the efficient removal of superoxide anions at their site generation throughout the cell. SODs constitute the first enzymatic line of defense against ROS by catalyzing the dismutation of O_2^- to H_2O_2 . Different SOD isoenzymes, whose metal cofactor differs, have been identified. Superoxide dismutases are present in all subcellular compartments susceptible to oxidative stress (Blokina et al., 2003). Our results indicate increased activity of one SOD isoenzyme (SOD 1) while maturity advanced as a response to the increment in the O_2^- level. However, the product of SOD activity, that is H_2O_2 , was not detectable or was found at very low levels at harvest in the maturity stages evaluated.

CAT, APX and POD are enzymes capable of scavenging H_2O_2 . The affinity of these enzymes for H_2O_2 is different. PODs are a large family of enzymes that can act as ROS scavengers. The optimal substrate for many of them is hydrogen peroxide, but others are more active with organic

hydroperoxides, such as lipid peroxides. CAT is present only in peroxisomes and proves essential when the peroxisome is stressed to detoxify the levels of ROS produced, while APX shows a high affinity for H₂O₂ and is important in abolishing H₂O₂ in all the cellular compartments (Mittler, 2002). The APX enzyme detoxifies peroxides, such as hydrogen peroxide, by using ascorbate as a substrate. In this study, the results obtained from harvested fruit suggest basal activity of CAT and POD during fruit maturation. Nevertheless, APX activity showed a gradual decline from stage SI to SIII. Del Bubba et al. (2009) reported a decrease in ascorbate content during 'Rojo Brillante' maturation owing to fruit growth rather than to a degradation process. A progressive reduction in the availability of the substrate for the APX enzyme would explain its diminished activity observed in this study during fruit maturation. APX was also seen to gradually decrease during ripening tomato (Mondal et al., 2004) but, in this case, it was not associated with lower ascorbate levels.

By taking together the SOD activity results, which showed an increment during maturation, and those of the H₂O₂ scavenging enzymes (stable POD and CAT activity, but diminished APX activity), we would expect H₂O₂ levels that are not only detectable, but which also increase from stage SI to stage SIII. However, the H₂O₂ levels were undetectable in the three studied maturity states. Contrarily to O₂⁻, peroxide is a not charge molecule that can diffuse to the vacuole where tannins are located (Mullineaux et al., 2000). Tannins possess an ideal structural chemistry for free radical scavenging activity, and they may be also play an important role in H₂O₂ scavenging. It has been shown that phenolic compounds may be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki, 1997).

The study of fruit after being submitted to CO₂ deastringency treatment has revealed that in all the maturity stages under study, CO₂ treatment results in a drastic drop in soluble tannins with values of 0.03%fw, which are habitual for non astringent 'Rojo Brillante' (Salvador et al., 2007). As previously outlined, the insolubilization of tannins is mediated by acetaldehyde, which is generated under anaerobic conditions (Matsuo and Ito, 1982). Accordingly, deastringency treatment led to a sharp rise in the acetaldehyde content in all the studied maturity stages. In parallel to the insolubilization of tannins after the deastringency process, antioxidant capacity drastically lowered, which confirms the link between soluble tannins content and the total antioxidant capacity of fruit.

Ethylene production was triggered after applying CO₂ treatment to the fruit in stages SI and SII, but it was not detected in maturity stage SIII. CO₂ increased after treatment for all three maturity stages. Despite deastringency treatment affecting the ethylene level, no significant effect was observed for the firmness and color values. We must bear in mind that physiological measurements were taken 1 day after treatment. A slight advance in color and fruit softening 3 days after treatment has been reported (Salvador et al., 2007).

The measurements of the O₂⁻ and H₂O₂ levels taken after deastringency treatment revealed that the accumulation of both species considerably changed as compared to that at harvest.

For all the maturity stages, O₂⁻ concentration increased after treatment to 2.5 nmol g⁻¹, regardless of the initial level at harvest. Excessive ROS generation (i.e., under oxidative stress) is an integral part of many stress situations, including hypoxia (Blokhina et al., 2003). ROS generation mechanisms in biological systems are represented by both non enzymatic and enzymatic reactions. Xanthine oxidase (XOD) is a key enzyme that is responsible for initial dioxygen activation. Xanthine oxidase can use xanthine, hypoxanthine or acetaldehyde as electron donors (Bolwell and Wojtaszek, 1997). Hence, acetaldehyde is a possible source of hypoxia-stimulated ROS production; Gong and Mattheis (2003) studied 'Braeburn' apple browning under low-oxygen and anaerobic conditions to report that addition of exogenous acetaldehyde before fruit storage resulted in enhanced XOD and NADH oxidase activity, and in reduced SOD activity, leading to superoxide accumulation.

Therefore, the increased O₂⁻ level observed in the present research work after treating fruit for deastringency may be associated with the drastic rise in acetaldehyde linked to the anaerobic respiration of fruit. However unlike what Gong and Mattheis (2003) reported, our results indicate that SOD activity was up-regulated after anaerobic treatment. Isoenzyme SOD 1 activity clearly enhanced after treatment. Moreover the appearance of another isoenzyme, SOD 2, was observed, but it was not detected at harvest. The appearance of other isozymes under anoxia conditions has been previously observed in plants (Biemelt et al., 2000), but to our knowledge, this is the first report in fruit. SOD isoenzymes are nuclear-encoded, and SOD genes in plants have been seen to be sensitive to environmental stresses, presumably as a result of increased ROS formation (Blokhina et al., 2003).

The rise in SOD activity induced by CO₂ treatment led to H₂O₂ accumulation after deastringency, which was more accused as maturation advanced. H₂O₂ accumulation associated with enhanced SOD activity under hypoxic conditions has been reported in the roots and leaves of *Hordeum vulgare*, and also in wheat roots (Biemelt et al., 2000; Kalashnikov et al., 1994).

The enzymatic assays revealed that CO₂ treatment enhanced H₂O₂ scavenging enzymes, besides SOD activity, in response to the rise in the H₂O₂ level. CAT activity increased after treatment in all the maturity stages, but more markedly in stages SI and SIII. APX activity was greater after treating fruit with CO₂ throughout the maturation period. Similarly to the pattern observed at harvest, APX reached higher post-deastringency levels with more immature fruit. Yet the extent of the increment observed for the values taken at harvest was greater in stage SIII than in the earlier stages; that is, APX activity in the stage SI CO₂-treated fruit increased by 2-fold as compared to the harvest values, but increased by around 4-fold in the stage SIII fruit.

The effect of CO₂ treatment on POD enzyme activity was found to be clearly dependent on the maturity stage at harvest. The activity of this enzyme was not affected in fruit in stages SI and SII. However, its level increased sharply when deastringency treatment was applied to the stage SIII fruit. POD activity showed a positive correlation with the H₂O₂ levels after treatment, suggesting that increased POD activity is linked to excessive H₂O₂ accumulation.

The increase in ROS scavenging enzymes activity observed after deastringency treatment agrees with that reported by Blokhina et al. (2003), who described an up-regulation of the antioxidant system when oxygen was lacking in plants.

In the present study, the effect of CO₂ treatment on both the levels of the ROS and ROS scavenger enzymes depended on the fruit maturity stage at harvest to some extent. While a similar response was observed in fruit in maturity stages SI and SII, the stage SIII fruit presented the highest levels of H₂O₂ and POD after deastringency, and displayed the most marked increase in APX activity. The stage SIII fruit also showed the greatest O₂⁻ accumulation and the poorest APX activity at harvest. Accordingly, the initial redox state of fruit at harvest seems to affect the stress response induced by CO₂ treatment.

Inducing oxidative stress by CO₂ deastringency treatment in persimmon fruit reported herein might explain the effect of this treatment on some physiological parameters previously described. So accumulation of O₂⁻ and H₂O₂ in fruit flesh after CO₂ treatment probably plays a key role in the degradation of the cell membranes associated with the CO₂ treatment described by Salvador et al. (2007). Lipid peroxidation is a natural metabolic process which, under normal aerobic conditions, is one of the most investigated consequences of ROS action on membrane structure and function. Polyunsaturated fatty acids, the main components of membrane lipids, are susceptible to peroxidation, and the initial phase includes activation of O₂ (Blokhina et al., 2003). Fruit softening on day 3 post-deastringency has been reported (Salvador et al., 2007), and reduced flesh firmness associated with CO₂ treatment duration when prolonged up to 40 h has been described.

Another previously reported effect of CO₂ treatment is an increase in the level of lipid-derived aldehydes as compared to those detected at harvest (Besada et al., 2012). An enhanced oxidative environment in fruit as a result of applying deastringency treatment could explain the enhancement of volatile compounds associated with the lipid oxidation process.

Besides, Del Bubba et al. (2009) reported an increment in the reduced ascorbate form *vs.* the oxidized one in CO₂-treated fruit when compared to fruit at harvest. This fact may form part of the defense response to oxidative stress since one research work done on the antioxidant defense system in the roots of wheat seedlings under root hypoxia or whole plant anoxia revealed a significant increase in the reduced forms of ascorbate and glutathione (Biemelt et al., 1998).

5. Conclusions

To summarize, the present paper reports that CO₂ treatment which is commonly employed to remove astringency from persimmon, leads fruit to an oxidative stress state; changes in both the ROS levels and the activity of the main ROS scavenging enzymes are described. The knowledge acquired herein can be useful to understand the physiological changes induced by this treatment in previous studies. Moreover, it is reported that the ripening process in persimmon may be linked to redox changes in fruit for first the time.

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CHAPTER IV

Involvement of the Redox System in Chilling Injury and Its Alleviation by 1-Methylcyclopropene in ‘Rojo Brillante’ Persimmon

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Abstract

A treatment with 1-methylcyclopropene (1-MCP) is known to reduce softening to the flesh of 'Rojo Brillante' persimmon, which is the main chilling injury symptom that occurs after storage at low temperature. However, very little is known about the mechanism by which 1-MCP confers persimmon tolerance to chilling. The aim of this study was to investigate the changes in the redox system associated with chilling injury and its reduction by 1-MCP during storage at 1 °C and after shelf-life period. Our results showed that during cold store, both control and 1-MCP treated fruit underwent gradual oxidative stress (accumulation of H₂O₂, increment in APX, CAT, LOX and slight increase in SOD activity) but no chilling injury was manifested. During shelf-life conditions, ethylene production was slightly higher in control than in 1-MCP treated fruit. Besides, the chilling injury manifestation of control fruit was associated with oxidative burst (major H₂O₂ accumulation and sharp increase in catalase (CAT), peroxidase (POD) and lipoxygenase (LOX) activity), while 1-MCP treatment greatly reduced the chilling injury symptoms. The 1-MCP treated fruit showed down-regulated POD activity and up-regulated CAT activity, which resulted in slower H₂O₂ accumulation. The reduction of the flesh softening as the main manifestation of chilling injury in 'Rojo Brillante' persimmon by 1-MCP was associated with the modulation of the redox state of the fruit during the shelf-life period that follows low-temperature storage.

Keywords: Flesh softening, Oxidative stress, Catalase, Peroxidase, Hydrogen peroxide

Introduction

Persimmons, like most of tropical and subtropical fruits are sensitive to chilling injury (CI), which is mainly expressed by flesh gelling (development of a gel-like consistency in the flesh), fast fruit softening and flesh browning during and after storage. The CI symptoms as well as their incidence and severity depend upon the cultivar, the storage temperature and duration. Besides, the CI symptoms became most severe after transferring fruit from low to ambient temperatures, although they can also be exhibited during cold storage (Woolf et al., 1997; Zhang et al., 2010).

'Rojo Brillante' persimmon has been widely reported to exhibit chilling injury symptoms when stored at temperatures below 15 °C. The main CI symptom of this cultivar is a fast firmness loss. This flesh softening can be exhibited during cold storage at 4 to 11 °C (Arnal and Del Río, 2004; Orihuel-Iranzo et al., 2010), nevertheless at 0 to 1 °C the drastic firmness loss only occurs when fruit are transferred to shelf-life temperatures (Arnal and Del Río, 2004; Salvador et al., 2004). Therefore the use of treatments to control chilling injury becomes necessary to cold storage persimmon. In this way 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, has been shown to reduce CI symptoms in a large number of persimmon cultivars, including 'Rojo Brillante' (Girardi et al., 2003; Kim and Lee, 2005; Salvador et al., 2004; Tibola et al., 2005).

Studying CI in persimmon and its reduction by 1-MCP has been widely addressed from the changes in flesh structure perspective. Thus Luo and Xi (2005) reported in chilling injured fruit that the primary cell wall and the middle lamella cannot be dissolved normally, while De Souza et al. (2011) reported an increase in the activity of cell wall-degrading enzymes such as endo-1,4- β -glucanase, pectin methylesterase, polygalacturonase and β -galactosidase. In 'Rojo Brillante' persimmon, microstructural studies have shown that 1-MCP preserves the integrity of cell walls and adhesion between adjacent cells (Pérez-Munuera et al., 2009). Moreover the loss of the cell walls integrity and the flesh breakdown associated with CI development has been linked to increased levels of ethylene (De Souza et al., 2011; Luo and Xi, 2005; Orihuel-Iranzo et al., 2010; Woolf et al., 1997). According to Orihuel-Iranzo et al. (2010), the increase in ethylene production upon transfer from chilling to nonchilling temperature is part of the fruit chilling response since it does not occur at

nonchilling temperatures, and it also appears to be responsible for the collapse of the fruit.

In addition to the activity of cell wall-degrading enzymes, cell structure can also be disrupted as a result of peroxidation caused by excess reactive oxygen species (ROS) (Blokhina et al., 2003). The ROS metabolism is controlled by an array of interrelated enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), which act concomitantly with non enzymatic antioxidants; O_2^- is converted into H_2O_2 by SOD, while H_2O_2 is scavenged predominantly by APX, CAT and POD. The implication of the oxidative system in CI sensitivity has been studied in depth in other fruits, in which chilling manifestation and its reduction by different treatments have been related to changes in ROS and enzymes of the oxidative system. This is the case of loquats (Cao et al., 2009, 2011; Wang et al., 2005; Xu et al., 2012), apple (Rupasinghe et al., 2000) or mandarins (Sala, 1998; Sala and Lafuente, 1999, 2000).

In 'Fuyu' persimmon, Zhang et al. (2010) described changes in the oxidative system during low temperature storage associated with reduced CI by means of 1-MCP. In this study the reduction by 1-MCP of chilling injury symptoms; the external and internal damages exhibited during storage at 4 °C, was attributed to altered oxidative status during cold storage. It must be taken into account that in the case of 'Rojo Brillante' the main symptom of CI is the drastic flesh softening, and it is not exhibited while fruit is stored at low temperature (1 °C) but is displayed after transferring fruit to shelf-life conditions. Besides this CI symptom is likewise reduced by 1-MCP when it is applied before or after cold storage. Therefore, the involvement of the redox system in chilling injury and its reduction by 1-MCP in 'Rojo Brillante' should be approached covering both low temperature storage and subsequent shelf-life period.

This study aims to investigate changes during cold storage and shelf-life period in the oxidative system (H_2O_2 content, SOD, CAT, APX, POD and lipoxygenase (LOX) enzymes activity) associated with CI reduction by 1-MCP in 'Rojo Brillante'.

Material and Methods

Plant material. Persimmon (*Diospyros kaki* Lf.) cv. Rojo Brillante fruit were harvested in l'Alcúdia (Spain) at mid-season. After harvest, fruit were taken to the Instituto Valenciano de Investigaciones Agrarias (IVIA), where they were divided into 20 homogenous lots of 15 fruit. One lot of fruit was analyzed to determine the maturity stage of fruit at harvest (color index of 9.42, firmness of 39.25 Newton (N) and soluble tannins content of 0.5 % FW). The remaining lots were submitted to a postharvest deastringency treatment with CO₂ under standard conditions (95 % CO₂, 24 h, 20°C, 90 % RH) in closed containers. After the deastringency treatment, fruit were kept at ambient temperature in an air atmosphere for 24 h. After this time, the physiological and redox state of fruit was evaluated with one lot of fruit.

The remaining 18 lots of fruit were divided into two groups of nine lots each in order to submit one of them to 1-MCP treatment (500 nL L⁻¹ of 1-MCP for 24 h at 20°C), while the other group acted as the control. Fruit from each treatment were stored at 1 °C, 85-95 % relative humidity (RH) for up to 45 days. Periodically (15, 30, and 45 d), three lots of 15 fruit per treatment were removed from the storage room. One lot was analyzed directly and the two other lots were transferred to 20°C to simulate the shelf-life conditions. After 2 and 5 days at 20°C, one lot of fruit was analyzed.

The evaluation of fruit immediately before storage (24 h after being submitted to CO₂) and periodically during storage and the subsequent shelf-life periods involved the determination of physiological parameters (firmness, color, soluble tannins content) and the redox state of fruit (hydrogen peroxide concentration (H₂O₂) and the activity of the SOD, CAT, POD, LOX and APX enzymes). CO₂ and ethylene production were also evaluated on days 1, 2 and 5 of each shelf-life period.

1-MCP (SmartFresh™), provided by 'AgroFresh' Inc. (Rhom and Haas Inc., Gessate, Italy), is formulated as a powder (0.14 % 1-MCP) and it was applied in closed chambers. The calculated quantity of SmartFresh needed to obtain the required 1-MCP concentration in each chamber was placed in a 125-mL tight-sealed bottle, and warm water (16 mL g⁻¹ product) was added through the septum. It was shaken in a warm water bath until turbidity had disappeared (~40 min). The sealed bottles were put inside each 442-L chamber and were

opened just before closing it. After 24 h, the chambers were opened and the fruit from each treatment were stored at 1°C and 85 to 95 % RH for up to 45 days.

Evaluation of physiological parameters. Skin color was determined over 15 fruit using a Minolta colorimeter (Model CR-300 Ramsey, NY, USA). Hunter parameters 'L', 'a', 'b', were measured and the results were expressed as Color Index=1000a/Lb according to Jimenez-Cuesta et al. (1981). This Color Index, originally developed for citrus fruit, it has been shown to acuity reflect the skin color changes of persimmon fruit. Negative values indicate green tones and positive values indicate orange and red tones (Salvador et al., 2004; Salvador et al., 2007). Two measurements were performed on opposite equatorial area in each fruit.

Flesh firmness was evaluated with a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA, USA) using an 8-mm plunger. Fruit firmness values were the average of 12 fruit per treatment. The results were expressed as load in Newton (N) to break flesh on one side of fruit after removing peel.

Immediately after the firmness measurement, flesh samples were taken from the opposite side of six fruit and were frozen at -20°C until the soluble tannins analyses. The samples from six other fruit were cut into small pieces and frozen with liquid nitrogen to be ground and kept at -80°C until the analyses of H₂O₂ content and enzymes activity evaluation were done. In both cases, flesh samples were taken together from two fruit (three replicates, two fruit per replicate).

Soluble tannins were evaluated by the Folin-Denis method described by Taira (1995) and modified by Arnal et al. (2004). The results were expressed as a percentage of fresh weight (FW).

For determination of CO₂ and ethylene production, three fruit were weighed and individually sealed in 1-L glass jars for 2 h at 20°C, and 1 mL of headspace was analyzed in a Perkin Elmer gas chromatograph, equipped with a Poropak QS 80/100 column. To determine CO₂, a thermal conductivity detector was used. Helium was the carrier gas used at 9.2 psi. The injector, oven and detector temperatures were 115°C, 35°C and 150°C, respectively. To determine ethylene, a flame ionization detector was used. Helium was the carrier gas used at 8 psi. The injector, oven and detector temperatures were 175°C, 75°C and

175°C, respectively. CO₂ production was expressed as mmol CO₂ kg⁻¹ h⁻¹ and ethylene production as nmol C₂H₄ kg⁻¹ h⁻¹.

H₂O₂ content. Hydrogen peroxide was determined from frozen tissue from three replicates each of two fruit. The method used was that according to Novillo et al. (2014). First, 1 g of flesh tissue was homogenized in an ice bath with 1.5 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12000 rpm for 20 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). Supernatant absorbance was measured at 390 nm. Hydrogen peroxide content was determined using a standard curve and was expressed as nmol g⁻¹ FW.

Enzyme activity measurement. All the enzyme extraction procedures were conducted at 4°C from frozen tissue from three replicates each of two fruit. For CAT, POD and LOX, 0.5 g of frozen tissue was homogenized with 2.5 mL of 50 mM sodium phosphate buffer (pH 7) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) and 10 g L⁻¹ polyvinylpyrrolidone (PVP). For APX, the same buffer was used, but also contained 0.5 mM ascorbic acid (AsA). For SOD, 0.5 g of frozen sample was homogenized with 2.5 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC). Homogenates were centrifuged at 12000 rpm for 25 min at 4°C. Supernatants were used for the enzymes assays. For all the enzymes, activity was expressed in units per gram of FW per minute.

CAT activity was assayed according to Novillo et al. (2014). This involved monitoring the disappearance of H₂O₂ by recording the drop in absorbance at 240 nm of a reaction mixture containing 50 mM of sodium phosphate buffer (pH 7), 90 mM of H₂O₂ and 0.5 mL of the CAT extract. The molar extinction coefficient of H₂O₂ at 240 nm was taken as 40 mM⁻¹ cm⁻¹ according to Duan et al. (2011). One unit of CAT activity was defined as the amount of enzyme that decomposed 1 μmol of H₂O₂ min⁻¹.

APX activity was measured according to Novillo et al. (2014). Activity was assayed in a mixture containing 2 mL of sodium phosphate buffer (50 mM, pH 7), 1 mM of ascorbic acid and 0.5 mL of H₂O₂ (4 mM). The reaction was initiated by the addition of 0.5 mL of enzyme extract. APX activity was determined by monitoring the drop in absorbance at 290 nm as ascorbate was

oxidized. A molar extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ according to Duan et al. (2011) was used to calculate activity. One APX unit was defined as the amount of enzyme to cause a decrease in OD₂₉₀ per min under the assay conditions.

POD activity was determined by measuring the increase in absorption at 470 nm according to Novillo et al. (2014). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7), 90 mM of H₂O₂ and 2% guaiacol. The reaction was initiated by adding 0.3 mL of the POD extract. A molar extinction coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate activity (Duan et al., 2011). One POD unit was defined as the amount of enzyme to cause an increase in OD₄₇₀ per min under the assay conditions.

LOX activity was assayed by monitoring the formation of conjugated dienes from linoleic acid at 234 nm, according to the method of Zheng et al. (2007) with some modifications. Three milliliters of reaction mixture contained 2.425 mL of sodium phosphate buffer (50 mM, pH 7), 75 μL linoleic acid solution (10 mM) and 0.5 mL enzyme extract. The blank contained 2.925 mL of sodium phosphate buffer (50 mM, pH 7) and 75 μL of linoleic acid solution (10 mM). One unit of LOX was defined as the amount of enzyme to produce an OD₂₃₄ reduction per min under the assay conditions. A molar extinction coefficient of $25 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate activity (Quartacci et al., 2001).

SOD activity determination was measured by the method of Beauchamp and Fridovich (1971) with some modifications. The reaction mixture contained 850 μL of 50 mM sodium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid (DETAPAC), 2.24 mM nitrotetrazolium blue chloride (NBT) solution, 1.8 mM xanthine solution, 40 U/mL catalase from bovine liver, 50 μL tissue extract. The reaction was initiated by the addition of 100 μL xanthine oxidase and was carried out at 25°C for 60 min. Assay mixture absorbance was measured at 560 nm. An assay mixture without tissue extract was used as a control. One enzyme activity unit was defined as the amount of enzyme that inhibited the photoreduction of NBT by 50%.

Statistical analysis. Data were subjected to the analysis of variance, and multiple comparisons between means were determined by the least significant difference test ($P \leq 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA).

Results

In the present study, during the first 30 days of cold storage, the control and 1-MCP treated fruit showed no changes in firmness and external color if compared to the values recorded before storing. After 45 days of storage, a very slight decrease in firmness and increase in color were observed in all the fruit (Fig. 1A and 1B). When the control fruit were transferred to shelf-life conditions after 15 days of cold storage, they underwent drastic firmness loss from 35 N to 20 N and to 10 N after 2 and 5 days at 20°C, respectively. Faster softening was observed during the shelf-life period after 30 or 45 days of cold storage when fruit firmness dropped to values below 10 N within the first 2 days at 20°C. As expected, the 1-MCP treatment significantly reduced firmness loss after the different shelf-life periods. Thus, the 1-MCP treated fruit showed firmness close to 25 N after 2 days at 20°C, which followed at 15, 30 and 45 days of cold storage. After 5 days of shelf-life, the firmness value remained constant for the 15 and 30 days of cold storage, but lowered to 17 N when cold storage lasted 45 days (Fig. 1A).

In parallel to firmness loss, fruit exhibited increased external color when transferred from cold storage to the shelf-life conditions (Fig. 1B). Color evolution was retarded in the 1-MCP treated fruit if compared to the control fruit, but only during the shelf-life that followed the 45-day cold storage.

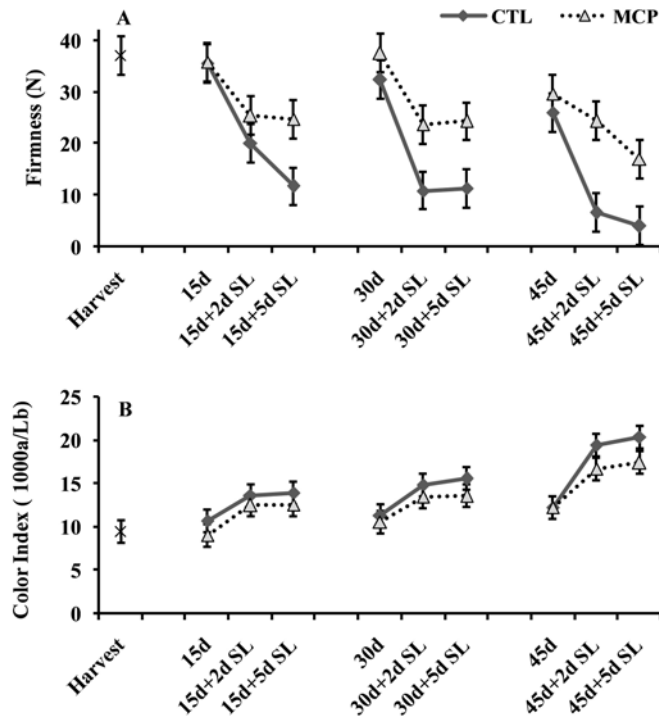


Fig. 1. Flesh firmness (A) and external color (B) of the untreated (CTL) and 1-methylcyclopropene-treated (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 day after deastringency treatment) and after 2 and 5 days of shelf-life at 20°C that followed to deastringency treatment plus 15, 30 and 45 days of cold storage at 1°C. Vertical bars represent the LSD intervals ($p < 0.05$).

The measurements of CO₂ and ethylene production taken during shelf-life that followed the different cold storage periods, showed that fruit firmness loss throughout storage was associated with an increment of ethylene and respiration rate after 1 day at 20°C (Fig. 2). The values of both these parameters gradually lowered after 2 and 5 days of shelf-life. In general, the fruit treated with 1-MCP showed a lower respiration rate than the control fruit. Ethylene production was also depressed by 1-MCP, which became evident during shelf-life after 30 and 45 days of cold storage.

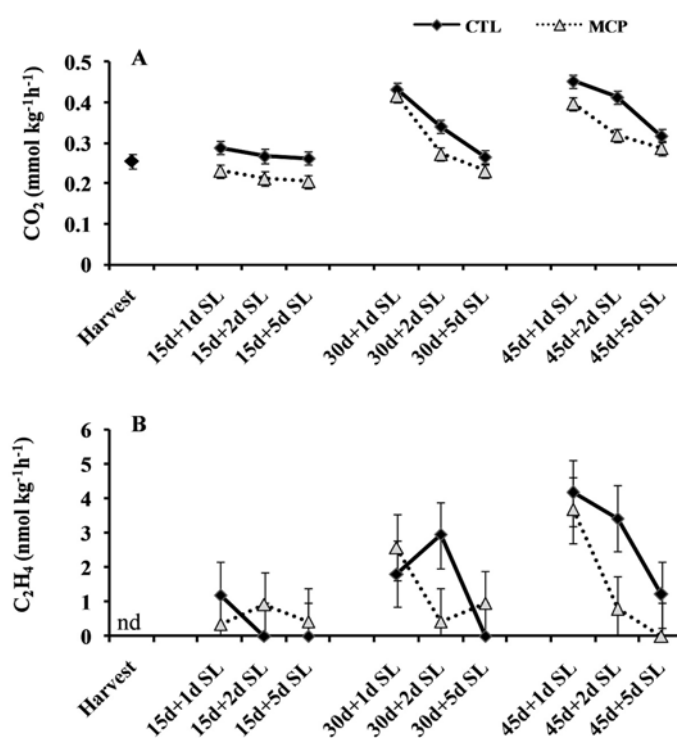


Fig. 2. Carbon dioxide (CO₂) (A) and ethylene (C₂H₄) (B) production of the untreated control (CTL) and the 1-methylcyclopropene-treated (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 day after destringency treatment) and after 1, 2 and 5 days of shelf-life at 20°C that followed to destringency treatment plus 15, 30 and 45 days of cold storage at 1°C. Vertical bars represent the LSD intervals ($p < 0.05$). *nd*: non detected.

During cold storage, no differences were exhibited in H₂O₂ content (Fig. 3) in either the pro- and antioxidant enzymes activity between the control and 1-MCP treated fruit (Figs. 4 and 5). After 15 days of cold storage, H₂O₂ content was slightly reduced (25 nmol g⁻¹) when compared to the initial content (40 nmol g⁻¹). However, a sharp increase to 70 nmol g⁻¹ of H₂O₂ was observed after 30 days of storage and then H₂O₂ content remained constant as storage advanced (Fig. 3). The trend of changes noted in APX activity ran in parallel to that observed for H₂O₂ content; APX activity declined from 1500 U g⁻¹ to 1000 U g⁻¹ during the first 15 days of storage. After 30 days, this activity reached

similar values to those observed at the beginning of storage and then remained unchanged after 45 days (Fig. 4A). CAT activity did not undergo major changes if compared to their initial values during 30 days of low-temperature storage, although this enzyme exhibited increased activity after 45 days (Fig. 4B). LOX activity enhanced from 1.1 U g^{-1} to 5.4 U g^{-1} during the first 15 days of cold storage and slightly increased throughout the storage (Fig. 4C). POD activity remained constant throughout the 45-day cold storage, with similar values to initial ones (25 U g^{-1}) (Fig. 5A). The SOD activity values did not change during the first 15 days of cold storage, and slightly increased after 30 days to remain at similar levels after 45 days (Fig. 5B). Therefore, the study of the redox system revealed some important changes in H_2O_2 content and pro- and antioxidant enzymes activity during cold storage, but no effect of 1-MCP treatment.

During the shelf-life that followed the cold storage periods, the 1-MCP treatment had no effect on APX, LOX and SOD activity (Fig. 4A, 4C, 5B). Both the control and 1-MCP treated fruit exhibited slightly reduced APX and SOD activity during 5 days at 20°C if compared to the values recorded after the cold storage periods. LOX enzyme activity sharply increased when fruit were transferred to shelf-life. While this increase in the cold-stored fruit over 15 days was observed only after 5 days at 20°C , when the storage period lasted 30 days and 45 days, this activity was enhanced after 2 days of shelf-life.

A major effect of 1-MCP treatment was observed on H_2O_2 content (Fig. 3) and on CAT and POD activity during shelf-life (Fig. 4B, 5A). A significant increase in H_2O_2 content was noted in the control fruit during 5 days at 20°C after the cold storage periods. However this increment was delayed in the 1-MCP treated fruit and even no changes in H_2O_2 content were seen in the 1-MCP treatment during shelf-life after 15 days of cold storage. CAT enzyme activity drastically increased during shelf-life after cold storage, especially after 15 and 30 days. Although a similar rise took place in both treatments when fruit were stored for 15 days, the 1-MCP treated fruit showed higher CAT activity values than the control fruit after shelf-life that followed 30 and 45 days of storage.

The effect of the 1-MCP treatment on POD activity during the shelf-life period was especially marked (Fig. 5A). While no major changes were observed in the 1-MCP treated fruit, POD activity in the control fruit tripled after shelf-life when compared to the values recorded at storage. This increase was

observed after 5 days of shelf-life when fruit were stored for 15 days, and after 2 days at 20°C when cold stored for 30 or 45 days.

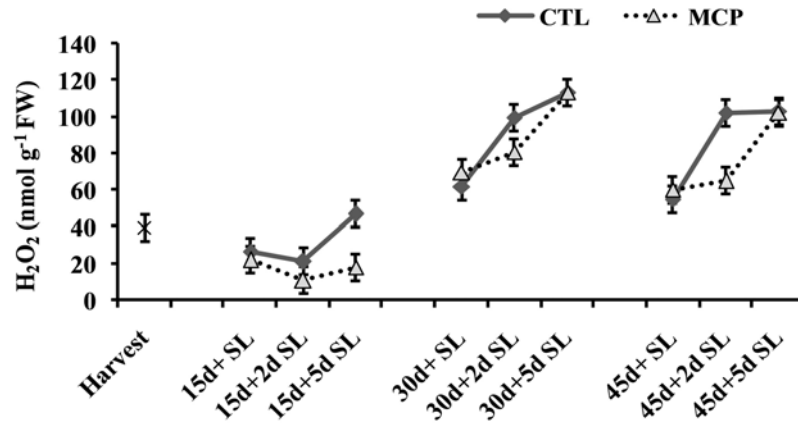


Fig. 3. Hydrogen peroxide (H₂O₂) content of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 day after destringency treatment) and after 2 and 5 days of shelf-life at 20°C that followed to destringency treatment plus 15, 30 and 45 days of cold storage at 1°C. Vertical bars represent the LSD intervals ($p < 0.05$).

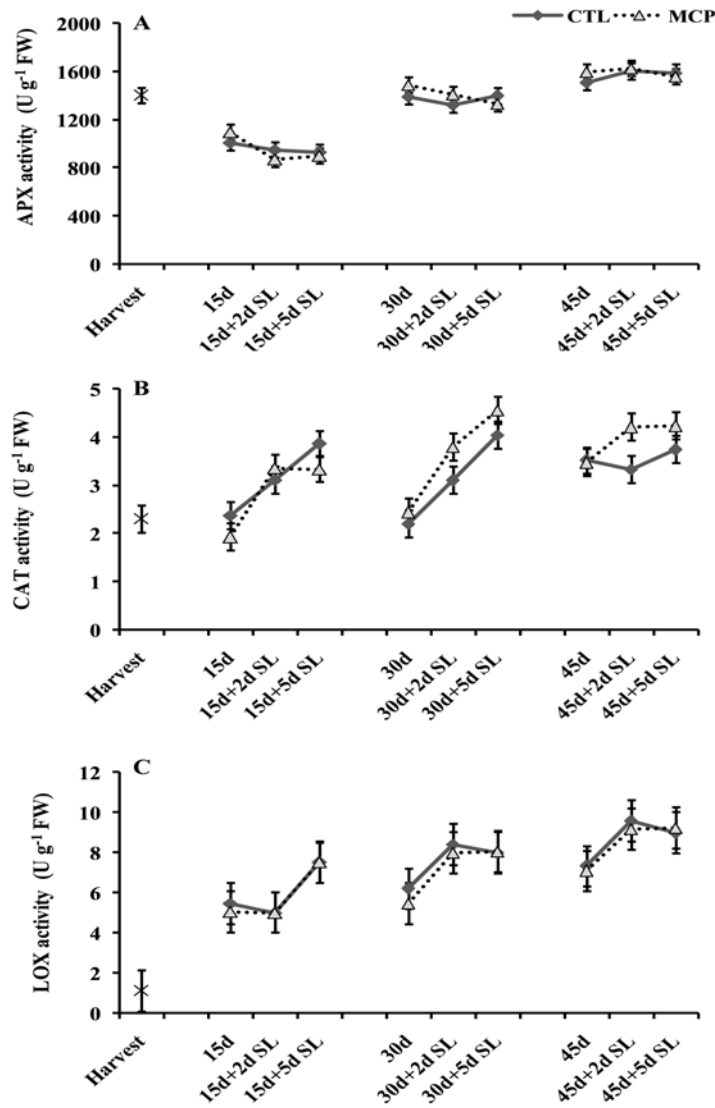


Fig. 4. Enzyme activity of ascorbate peroxidase (APX) (A), catalase (CAT) (B) and lipoxygenase (LOX) (C) of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) 'Rojo Brillante' persimmons at Harvest (1 day after deastringency treatment) and after 2 and 5 days of shelf-life at 20°C that followed to deastringency treatment plus 15, 30 and 45 days of cold storage at 1°C. Vertical bars represent the LSD intervals ($p < 0.05$).

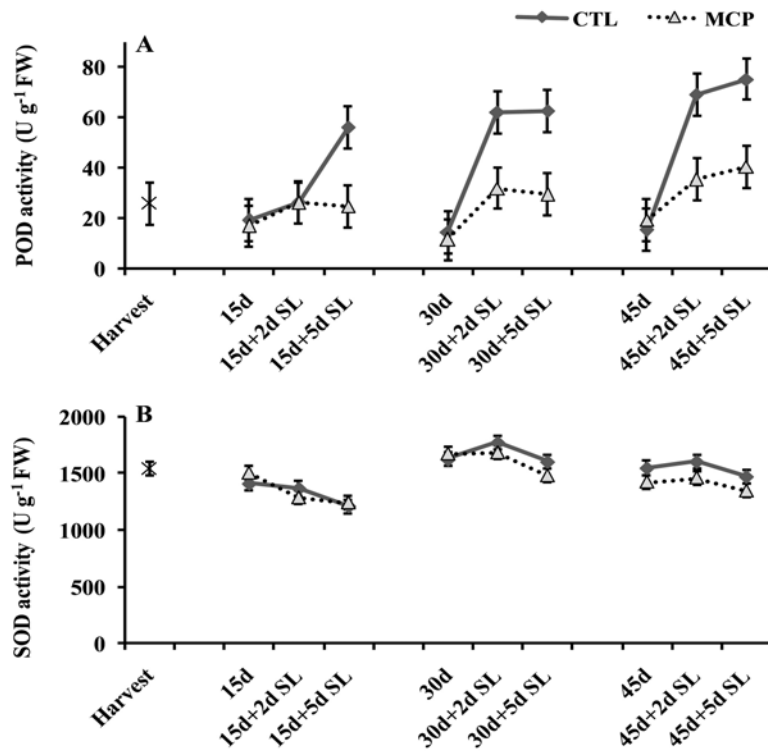


Fig. 5. Enzyme activity of peroxidase (POD) (A) and superoxide dismutase (SOD) (B) of the untreated fruit (CTL) and the 1-methylcyclopropene-treated fruit (1-MCP) ‘Rojo Brillante’ persimmons at Harvest (1 day after deastringency treatment) and after 2 and 5 days of shelf-life at 20°C that followed to deastringency treatment plus 15, 30 and 45 days of cold storage at 1°C. Vertical bars represent the LSD intervals ($p < 0.05$).

Discussion

The results obtained in the present study corroborate that the drastic fruit softening is the main CI symptom in 'Rojo Brillante' persimmon and is manifested only after fruit are transferred from low-temperature (1°C) storage to shelf-life conditions. Chilling damage became evident during shelf-life that followed to 15 days at 1°C, but it was aggravated by the cold-storage time. In accordance with previous results (Salvador et al., 2004), the 1-MCP treatment significantly reduced fruit firmness loss during shelf-life.

Despite no chilling damage being observed while fruit was maintained at low temperature, the redox stage of fruit underwent several important changes during this cold storage period. It is important to highlight that these changes were similar in control and 1-MCP treated fruit. Accordingly with numerous reports that support the idea that chilling stress increases ROS production, including O_2^- , H_2O_2 and OH^- (Sevillano et al., 2009), in the present study, after 30 days of cold storage a major H_2O_2 accumulation was observed. This increase in H_2O_2 content would respond to the slight increase in SOD activity detected, an enzyme responsible for converting O_2^- into H_2O_2 . Sala (1998) reported that chilling stress in cold-stored mandarins was associated with the activation of the antioxidant defense system in response to increasing prooxidant levels. In line with this, our results reveal that after declining the activity of APX at the beginning of the storage, it is up-regulated after 30 days of storage, while CAT activity importantly increases after 45 days if compared to the values recorded for shorter storage periods. The earlier response of the APX enzyme to H_2O_2 accumulation, if compared to the CAT enzyme, is explained by the higher affinity that APX shows for H_2O_2 . The APX enzyme is involved in abolishing H_2O_2 in all cellular compartments, while the CAT enzyme is present only in peroxisomes and is essential for detoxifying ROS levels when peroxisome is stressed (Mittler, 2002). Besides the activity of APX and CAT, the activity of LOX was also enhanced during 45 days of storage.

Therefore although any chilling injury symptoms is manifested in the fruit during cold storage, the changes observed in the oxidative system during this period (increased SOD activity, H_2O_2 accumulation and subsequent up-regulation of CAT, APX and LOX enzymes) reveal that the fruit underwent progressive oxidative stress associated with low-temperature exposure, which was not reduced by the 1-MCP treatment.

Contrarily to that observed at cold storage, during shelf-life the changes in the redox system were affected by the 1-MCP treatment. So, transferring fruit from low to shelf-life temperatures lead to an oxidative burst, manifested as a major H₂O₂ accumulation and a subsequent drastic increase in the activity of the CAT, POD and LOX enzymes. Nevertheless in 1-MCP treated fruit the increase of H₂O₂ levels was lower than in control and higher CAT activity and lower POD activity were observed.

Ethylene plays a role in chilling injury, where exogenous exposure aggravates chilling injury symptoms (Park and Lee, 2005; Besada et al., 2010), and ethylene production and respiration rate are higher in chilling injured fruit after removal from cold storage (Besada et al., 2010). The effect of the 1-MCP treatment on attenuating the POD up-regulation observed in the present study is probably mediated by its effect on declining ethylene production during shelf-life, which is in accordance with different studies that reports the inductive action that ethylene exerts on POD. So, the induction of POD isoenzymes during ethylene-induced senescence is a common response in cultivars of *Cucumis sativus*, other species of *Cucumis* and other genera of Cucurbitaceae (Abeles et al., 1989). Similarly in climacteric fruit, POD and indoleacetic acid (IAA) oxidase isoenzymes have been reinforced with progressing maturity. However in non-climacteric fruits, where ethylene did not notably change during ripening, only the IAA oxidase isoenzyme concentration increased, while the POD isoenzyme concentration decreased (Vamos-Vigyazo, 1981). Furthermore in bamboo shoots, ethylene has been reported to increase POD activity, while 1-MCP treatments retarded it (Luo et al., 2008).

Moreover, the increased H₂O₂ concentration observed during shelf-life while SOD activity remained stable, or even declined, if compared to the values at cold storage, suggests that when fruit are transferred to moderate temperatures, a source of H₂O₂ not linked to SOD activity may exist. Several studies have reported H₂O₂ formation under different stresses as a result of peroxidase activity through a complex reaction with NADH which is oxidized using trace amounts of H₂O₂ first produced by the non enzymatic breakdown of NADH (Bestwick et al., 1998; Blokina et al., 2003; Kim et al., 2010; Simonovicova et al., 2004). This justify the unexpected results obtained in the present study where, under shelf-life conditions, control fruit showed an up-regulation of POD activity in parallel to an increase in H₂O₂ content, while the 1-MCP treatment delayed H₂O₂ accumulation, which is associated with lower POD activity levels.

Therefore, in 'Rojo Brillante' persimmon, development of CI symptoms during shelf-life is associated with a high POD activity and the fast H₂O₂ accumulation occurred when fruit is transferred from low to moderate temperature. In contrast, the slower H₂O₂ accumulation detected in the 1-MCP treated fruit, as result of greater CAT activity and the inhibition of POD activity, probably confers chilling tolerance to tissues. Similar results have been found by Wang (1995), who reported that the temperature-preconditioning treatment of zucchini squash reduced declining CAT activity and suppressed increased POD activity during cold storage, thus contributing to CI tolerance.

Moreover, the changes observed by Zhang et al. (2010) when studying the reduction in CI symptoms by 1-MCP on 'Fuyu' persimmon also support the fact that POD and CAT are key enzymes involved in the chilling sensitivity of persimmon. So, in this study, CI is manifested in 'Fuyu' during low-temperature storage and throughout cold storage POD activity increased by 4-fold in control fruit, while it was significantly inhibited in the 1-MCP treated fruit; besides, CAT activity was enhanced in 1-MCP treated fruit when compared to control fruit (Zhang et al., 2010). In the present study, neither POD nor CAT activity in 'Rojo Brillante' showed differences between control and 1-MCP treated fruit during cold storage. Both enzymes were up-regulated when fruit were transferred to shelf-life and chilling symptoms were manifested; the CI reduction by 1-MCP was related to declined POD activity and enhanced CAT activity during shelf-life if compared to control fruit.

The results of the present study allow us to understand why in 'Rojo Brillante' persimmon the CI reduction by 1-MCP treatment, which occurs during shelf-life, is similar when the 1-MCP application is performed before cold storage or immediately before transferring fruit to shelf-life conditions (Salvador et al., 2004).

In conclusion, our results demonstrate that the low-temperature storage of 'Rojo Brillante' persimmon leads to gradual oxidative stress, which is aggravated after 30 days of cold exposure and is not alleviated by 1-MCP. The manifestation of CI symptoms when control fruit were transferred from low temperatures to moderate ones is associated with an oxidative burst, with major H₂O₂ accumulation, and also with a sharp increase in CAT, POD and LOX activity. The reduction of CI symptoms by the 1-MCP treatment is linked to lower POD activity levels and enhanced CAT enzyme activity, which result in slower H₂O₂ accumulation.

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Chapter IV

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***III.2. EVALUATION OF POSTHARVEST
TREATMENTS TO IMPROVE FRUIT
QUALITY DURING COLD STORAGE***

CHAPTER V

Effect of a low oxygen atmosphere combined with 1-MCP pretreatment on preserving the quality of ‘Rojo Brillante’ and ‘Triumph’ persimmon during cold storage

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Abstract

For the purpose of prolonging the storage of 'Rojo Brillante' and 'Triumph', the main persimmon varieties cultivated in Spain, the effect of a controlled atmosphere based on 4-5% O₂ + N₂, applied alone or in combination with 1-MCP pretreatment, was evaluated herein after storage at 1°C for up to 90 days and subsequent simulated shelf-life conditions at 20°C. Our results showed for the first time that 'Triumph', a cultivar hitherto considered insensitive to chilling injury when cultivated under Spanish conditions, manifests flesh gelling and drastic fruit softening during cold storage, both of which are important symptoms of chilling injury in persimmon. 'Rojo Brillante' and 'Triumph' fruit showed different responses to the controlled atmosphere (CA) conditions studied. For 'Rojo Brillante', the combination of CA and 1-MCP pretreatment substantially alleviated chilling injury (flesh softening and gelling). However, all the fruit stored under CA, pretreated with 1-MCP or not, manifested internal flesh browning. For 'Triumph' fruit, the combined use of 1-MCP pretreatment and CA storage retarded fruit softening and markedly alleviated flesh gelling. This treatment was not associated with manifestations of internal disorders, and helped extend the storage period up to 90d, plus 5d at 20°C. CA-exposed fruit also displayed considerably fewer black spots caused by *Alternaria alternata*. Therefore, although the 1-MCP pretreatment and CA storage combination based on 4-5% O₂ + N₂ must be considered a useful technology to prolong the storage of 'Triumph' as it preserves internal and external fruit quality, it is necessary to optimise the appropriate CA storage conditions for 'Rojo Brillante'.

Keywords: 1-Methylcyclopropene, Chilling injury, Firmness, Flesh gelling, Flesh browning

1. Introduction

In the Mediterranean region, 'Rojo Brillante' and 'Triumph' are the main persimmon cultivars, and their production has considerably increased in the last decade. Both cultivars belong to the astringent cultivars group; therefore, astringency must be removed before commercialisation. Deastringency treatment based on high CO₂ exposure is currently applied after storage to market high texture fruit without astringency (Salvador et al., 2007; Tsviling et al., 2003). The commercial importance of these persimmon cultivars implies having to optimise storage technology. For 'Rojo Brillante', as with most persimmon cultivars, low-temperature storage is limited by the high chilling injury (CI) sensitivity, manifested as major flesh softening when fruits are transferred from low to shelf-life temperatures (Arnal and Del Río, 2004a; Besada et al., 2008; Salvador et al., 2004). Although 'Triumph' has been reported as a non-sensitive cultivar to CI, long cold storage is also limited by flesh softening which occurs during shelf-life (Tsviling, 2003; Woolf and Ben-Arie, 2011). Therefore, in Spain, the low-temperature storage of 'Rojo Brillante' and 'Triumph' fruits is currently mediated by the application of 1-methylcyclopropene (1-MCP) (commercially applied by Agrofresh as SmartfreshTM), whose positive effect on alleviating CI symptoms in persimmon has been widely reported (Besada et al., 2008; Pérez-Munuera et al., 2009; Salvador et al., 2004; Tsviling et al., 2003). This treatment helps prolong the cold storage of 'Rojo Brillante' and 'Triumph' to up to 30 and 60 days, respectively, to maintain internal and external quality. Yet, under our commercial conditions, it would be interesting to have other technology which, either alone or combined with 1-MCP, can help prolong storage.

Storage in a controlled atmosphere (CA) has become a common technology to prolong cold storage, while preserving the quality of fruits such as pears, apples and other commodities (Kader, 2002). In persimmon fruit, the effect of CA on extending storage has been widely studied in the non-astringent cultivar, 'Fuyu'. Burmeister et al. (1997) reported that atmospheres based on 100% N₂ or 5-10% CO₂ + 2% O₂ reduce CI in 'Fuyu', but lead fruit to manifest external browning. Later, a range of atmospheres was evaluated on the same cultivar by Donazzolo and Brackmann (2002), Brackmann et al. (2006) and Lee et al. (2003) among others. Currently, the incidence of skin and flesh disorders is the main limitation to store 'Fuyu' in a CA. So after studying the effect of different CO₂ and O₂ atmosphere conditions, Park and Lee (2008) reported the incidence of different types of skin and flesh browning on cold-stored 'Fuyu'. These authors reached the conclusion that such disorders are due mainly to low

levels of O₂, and not to high levels of CO₂. Storing 'Fuyu' in flowing nitrogen (<0.1% O₂) at 0°C has been shown to alleviate CI in storage for up to 8 weeks, but it may result in skin browning (Woolf and Ben-Arie, 2011).

To date, the study of CA storage has been limited for 'Rojo Brillante'. In 2008, we reported that during storage at 15°C, an atmosphere based on 97% N₂ + 3% air led 'Rojo Brillante' fruit to lose astringency and allowed fruit conservation for 30 days (Arnal et al., 2008). Recently, Orihuel-Iranzo et al. (2010) determined that ULO (ultra low oxygen) atmosphere (1.3-1.8% O₂) removed astringency in 'Rojo Brillante' when applied at 14.5°C, but it did not control CI at 1°C or 10°C. When a CA was applied to 'Triumph', CA (1-1.5% O₂ and 1.5-3% CO₂) storage of this cultivar has been reported to offer the benefit of delaying softening and retarding decay development. However, the fruit's shelf-life was inversely proportional to storage period length (Tsviling et al., 2003).

According to the information above, optimum CA conditions to prolong persimmon conservation do not seem to have been completely elucidated and still depend basically on variety. This is why a CA is rarely used commercially for persimmon. Therefore, the objective of this study was to determine the effect of a CA based on 4-5% O₂ + N₂, either alone or in combination with 1-MCP pre-treatment, on the quality of 'Rojo Brillante' and 'Triumph' persimmons stored at 1°C for up to 90 days.

2. Material and methods

2.1. Fruit source and storage procedure

Persimmon fruit of cvs. Rojo Brillante and Triumph were harvested in L'Alcudia (Valencia, east Spain) and transported to Tecnidex S.A.U. Co. (Valencia), where fruit were carefully selected for uniform size and external colour. Afterwards, fruits were separated into lots to be submitted to the following treatments: 1) Air: storage under air (acting as a control), 2) CA: storage in a CA (4-5% O₂ + N₂); 3) 1-MCP-Air: 1-MCP pretreatment + storage under air; 4) 1-MCP-CA: 1-MCP pretreatment + storage under CA (4-5% O₂ + N₂). Storage temperature was always maintained at 1°C, with RH at 85-90%. After 30, 60, 75, and 90 days of storage, two samples of 20 fruits from each treatment were transported to the Instituto Valenciano de Investigaciones

Agrarias (IVIA, Valencia). One sample was directly analyzed, while the other one was submitted to CO₂-deastringency treatment (95% CO₂ for 24 h at 20°C) and was then transferred to 20°C in an air atmosphere for 5 days to simulate the shelf-life period.

1-MCP (SmartFresh™), provided by AgroFresh Inc. (formulated as a powder; 0.14% 1-MCP) was applied in closed chambers at 1°C for 24 h. The calculated quantity of SmartFresh required to obtain a concentration of 1-MCP of 500 nL L⁻¹ in each chamber was placed in a 125-mL tight-sealed bottle, and warm water (16 mL g⁻¹ product) was added through the septum. It was shaken in a warm water bath until turbidity disappeared (~40 min). Sealed bottles were placed inside each chamber and were opened immediately before closing it. After 24 h, chambers were opened and the fruits from each treatment were stored at 1°C and 85-90% RH for up to 90 days.

The CA conditions were created using an N₂-generating system, which maintained O₂ concentration throughout the experiment at between 4-5% (v/v). The O₂ and CO₂ concentrations were periodically monitored by an automated system. The CO₂ concentration during the experiment was below 0.4%.

Deastringency treatment was carried out in closed containers which contained 95% CO₂ for 24 h at 20°C (90% RH) by passing an air stream containing 95% CO₂ through the containers.

2.2. Fruit quality assessment

After both storage periods at 1°C and the subsequent shelf-life, flesh firmness, external colour, acetaldehyde, ethanol, soluble tannins content (ST), sensory evaluation, and external and internal disorders were evaluated. Besides, decay incidence and severity was evaluated after 90 days of storage and the subsequent shelf-life period.

Flesh firmness was determined with 20 fruits per replicate in a texturometer (model 4301, Instron Corp., Canton, Mass., U.S.A.), using an 8-mm plunger after epicarp removal at two equidistant locations in the equatorial region of each fruit. The crosshead speed during firmness testing was set at 10 mm/min. Data were expressed as the maximum force in Newtons (N) required to break the flesh.

Soluble tannins content was evaluated by the Folin-Denis method, as described by Arnal and Del Rio (2004b), and was expressed as a percentage or g/100 g FW. As many as 15 fruit per replicate were divided into three samples and cut into four longitudinal parts. Two of the opposite parts were sliced and frozen (-20°C) to determine ST.

The opposite parts of fruit not used to measure ST were placed in an electric juice extractor (model 753, Moulinex, Spain) and filtered through cheesecloth; the obtained juice was used to determine acetaldehyde and ethanol production. Acetaldehyde and ethanol production were measured on three samples per replicate of the juice samples, obtained as mentioned earlier and analysed by headspace gas chromatography (Ke and Kader 1990). Five millilitres of the filtered juice were transferred to 10-mL vials with crimp-top caps, sealed with TFE/silicone septa, and frozen (-20°C) until analysis. For the analysis, samples were put in a water bath at 20°C for 1 h, followed by heating at 60°C for 10 min. A 1-mL sample of the headspace was withdrawn from the vials and injected into the gas chromatograph (model 2000, Perkin-Elmer, Norwalk, Conn., USA), provided with a flame ionisation detector (FID) and a 0.32 cm × 1.2 m Poropak QS 80/100 column. The injector was set at 175°C, the column at 150°C, the detector at 200°C, and the carrier gas at 12.3 psi. Ethanol and acetaldehyde were identified by comparison the retention times with those of a standard solution. The results were expressed as mg/100 mL.

Sensory evaluation was performed at the sensory Laboratory of the Postharvest Department (IVIA) in composite samples of five fruits from each replicate, which had been previously peeled and sliced. Eight to 10 semitrained panellists were asked to evaluate astringency and the presence of off-flavours. The panellists were familiar with the 'Rojo Brillante' and 'Triumph' cultivars, as they had been tasting fruits with different levels of astringency for several years. A 4-point scale was used for astringency, where 4=very high astringency and 1=no astringency. Each sample consisted of segments which were taken from about five individual fruits. Samples were presented to the panellists in trays labelled with 3-digit random codes and were served at room temperature. The panellists had to taste several segments of each sample in order to compensate, as far as possible, for the biological variation of the material. Milk was provided for palate rinsing between samples.

Skin colour was evaluated by a colorimeter (model CR-300, Minolta, Ramsey, N.Y., USA) on samples of 20 fruit. *L*, *a*, *b* Hunter parameters were

measured and the results were expressed according to the skin colour index $((1000a)/(Lb))$ (Salvador et al., 2007).

Flesh gelling, internal disorders and decay index were evaluated in four replicates of 5 fruit each one. Flesh gelling was assessed using methods modified from Woolf et al. (1997). Flesh gelling was rated on a 5-point scale according to the amount of gelling detected in the flesh of fruit cut through the equator: 0 = very firm fruit with little give, no gelling, flesh feels coarse when rubbed with finger; 1 = flesh with slight-moderate give, no gelling, feels slightly slippery to the touch; 2 = flesh with moderate give, slight gelling, feels slippery to the touch; 3 = flesh generally soft, gelling of 30-70% of cut surface, flesh slippery to touch; 4 = flesh soft, gelling of 70-100% of cut surface, flesh slippery to touch. In order to obtain a unique value that reflects both the incidence and severity of this disorder, the following flesh gelling index was calculated: $\Sigma ((\text{gelling severity}) \times (\text{number of fruits at each gelling severity})) / (\text{total number of fruits})$.

Internal disorders (flesh browning) were rated on a 4-point scale according to the intensity of the alteration: 0=absence; 1= slight; 2=moderate; 3=severe. Flesh browning index was calculated following the formula explained above.

Decay index was evaluated on a 6-point scale according to the severity of the disease: 0=absence of black spots, to 5-severe decay (more than 20% of the fruit surface affected by black spots). Decay index was calculated following the formula explained above.

2.3. Statistical analysis

Data were subjected to an analysis of variance (ANOVA). Multiple comparisons between means were made individually for each cultivar and period of evaluation with the LSD test ($P \leq 0.05$) using Statgraphics Plus 5.1 (Manugistics Inc., Rockville, Md., USA). Homoscedasticity of the data was checked by the Cochran's C test before ANOVA.

3. Results and discussion

3.1. Fruit firmness and flesh gelling related to chilling injury

The present study revealed a different pattern of CI manifestation during the cold storage and shelf-life of 'Rojo Brillante' and 'Triumph' fruits. The control 'Rojo Brillante' fruit (Air) maintained similar firmness values to those at harvest (40N) during the first 30 days of low-temperature storage (Fig. 1). After 60 and 75 days at 1°C, fruits showed slight softening (30N), and drastic loss of firmness was observed only after 90 days (10N). When fruits were transferred to 20°C after 30 d of storage, firmness dropped to 20N. However, when shelf-life was simulated after longer storage periods, the fruit drastically softened and showed firmness values lower than 10N. Flesh gelling (Table 1) appeared (a flesh gelling index of 1) in control fruit after a 60-day low-temperature storage and severity increased to 1.8 after 90 days. Flesh gelling was aggravated when fruits were transferred to shelf-life conditions. During the shelf-life that followed a 30-day storage, flesh gelling was slight, and was moderate during a shelf-life that followed longer storage periods. Accordingly, the main CI symptom reported in 'Rojo Brillante' persimmon was drastic fruit softening when fruits were transferred from a low to a moderate temperature (Arnal and Del Río, 2004a), and flesh gelling was observed only after prolonged storage periods.

Unlike 'Rojo Brillante', air-stored 'Triumph' fruits significantly softened from the start of the storage period (Fig. 1). Fruit firmness declined from 48N at harvest to 30N and 10N after 30 and 60 days at 1°C, respectively. When the control fruit were transferred to shelf-life conditions, flesh softening was aggravated, and firmness values dropped lower than 10N even after only 30 days. Apart from firmness loss, the untreated fruit of 'Triumph' manifested flesh gelling as a result of low temperature exposure. During cold storage, the longer the storage period, the worse this disorder became. In the control fruit, no flesh gelling was manifested after 30 days, but fruits exhibited a flesh gelling index of 1.4 after 60 days of storage, and this value increased to 2.3 after 90 d at 1°C. After shelf-life periods following low temperature storage, the flesh gelling disorder became more pronounced. It was manifested even after a shelf-life that followed 30-day storage (values of 1) and became severe (values of 4) when shelf-life periods were assayed after 60 days or longer storage periods. Flesh gelling has been reported to be the main CI in other cultivars, such as 'Fuyu,' in which CI has been studied in depth (Besada et al., 2010a; MacRae, 1987; Woolf et al., 1997). Despite 'Triumph' fruit having been reported as an insensitive

cultivar to CI (Woolf and Ben-Arie, 2011), our results indicate occurrence of flesh gelling during cold storage when this disorder is aggravated if fruit are transferred to shelf-life conditions. Flesh gelling also became more severe in 'Triumph' than in 'Rojo Brillante'. Therefore, our results strongly suggest that under our study conditions, 'Triumph' fruits manifested sensitivity to CI. Woolf and Ben-Arie (2011) reported a significant variation in the CI response of fruits from different countries. Hence, classic CI (gelling and loss of fruit juice) has been observed in 'Fuyu' in New Zealand, but not in Korea or Israel. The present study reveals that contrarily to that reported in other countries, the 'Triumph' fruits cultivated in Spain are sensitive to manifesting CI during low-temperature storage.

Regarding the effect of the CA and 1-MCP-Air treatments (Fig. 1), the fruits of both cultivars maintained similar and high firmness values close to 30N for 75 days at 1°C; nevertheless after 90 days, the 1-MCP fruit exhibited an important softening with firmness values similar to those of the control fruit, while the fruit subjected to a CA did not show this marked softening feature. After transferring fruit to the shelf-life conditions, as expected, the 1-MCP-treated 'Rojo Brillante' fruit showed high firmness values after 30 days (35N), and commercial values after 60 and 75 days (>10N). Likewise, the effect of 1-MCP was clearly observed in 'Triumph' fruits, which also presented commercial firmness after a shelf-life that followed 75 d cold storage. In addition, in the 1-MCP-treated fruits from both cultivars, flesh gelling was considerably alleviated compared to the control fruits. It is noteworthy that the fruit of both cultivars maintained in a CA displayed severe softening when transferred to the shelf-life conditions and showed similar firmness values to the controls. However, in both cultivars, the CA treatment delayed onset of flesh gelling alteration to 60 days of cold storage plus shelf-life, and its incidence was much less marked than in the control fruit.

The effect of CI alleviation by 1-MCP treatment on persimmon fruits has been extensively reported (Girardi et al., 2003; Salvador et al., 2004, Kim and Lee, 2005; Tibola et al., 2005; Tsviling et al., 2003). If it is considered that 10N is a firmness threshold for the commercialisation of persimmon fruits with a crisp texture, the CA treatment itself did not prolong the storage of either 'Rojo Brillante' or 'Triumph', because firmness loss was similar to that of the control fruit. Although the pretreatment with 1-MCP retarded fruit softening and helped extend the post-storage shelf-life of both cultivars up to 75d of storage (Fig. 1), specially in the case of 'Triumph', flesh gelling may become the storage limiting factor (Table 1; Fig. 2).

The response of both cultivars when CA storage was applied in combination with the 1-MCP pretreatment (1-MCP-CA) was significantly different. The 'Rojo Brillante' fruit maintained the same firmness values as those recorded at harvest (40N) during the 90-day storage at 1°C (Fig. 1). When fruits were transferred to the shelf-life conditions within a 75-day storage time, no firmness loss was detected, and only a slightly reduced firmness, with 30N values, was observed during the shelf-life that followed the 90-day storage. Furthermore, no flesh gelling was detected in the fruits pretreated with 1-MCP and stored in a CA.

In the 'Triumph' fruit, although the softening alleviation by 1-MCP followed by CA storage was less pronounced than in 'Rojo Brillante', this storage condition substantially reduced flesh gelling (Table 1). During low-temperature storage, the 'Triumph' fruits softened gradually and showed slightly higher firmness values than when only the CA treatment was applied (Fig. 1). Flesh gelling manifestation was delayed considerably and appeared only after 90 days at 1°C with a low incidence (value of 0.3). During the shelf-life periods, the benefit of MCP + CA alleviation of both softening and flesh gelling became even more evident. Thus, during the shelf-life that followed storages of 75 d and 90 d, the 1-MCP-CA-treated fruit showed the highest firmness values, 20N, and the lowest flesh gelling severity (<0.5) for the various assayed treatments.

Fruit softening alleviation after applying 1-MCP in combination with a CA based on 2.0 kPa of O₂ + free CO₂ atmosphere, has been reported for the cultivar 'Fuyu' during storage lasting 17 d at 10°C (Vilela et al., 2007). However, Brackmann et al. (2003) reported that the combined use of 1-MCP and CA (1kPa of O₂ + 5kPa of CO₂) for 2 months of cold storage of caqui cv. Quioto resulted in fruit with lower firmness values than when applying 1-MCP alone. A range of factors, such as atmosphere conditions, storage temperature and cultivar must be determinants for the response of fruits to not only CA storage, but also to the effect of combining this technology with a 1-MCP pretreatment.

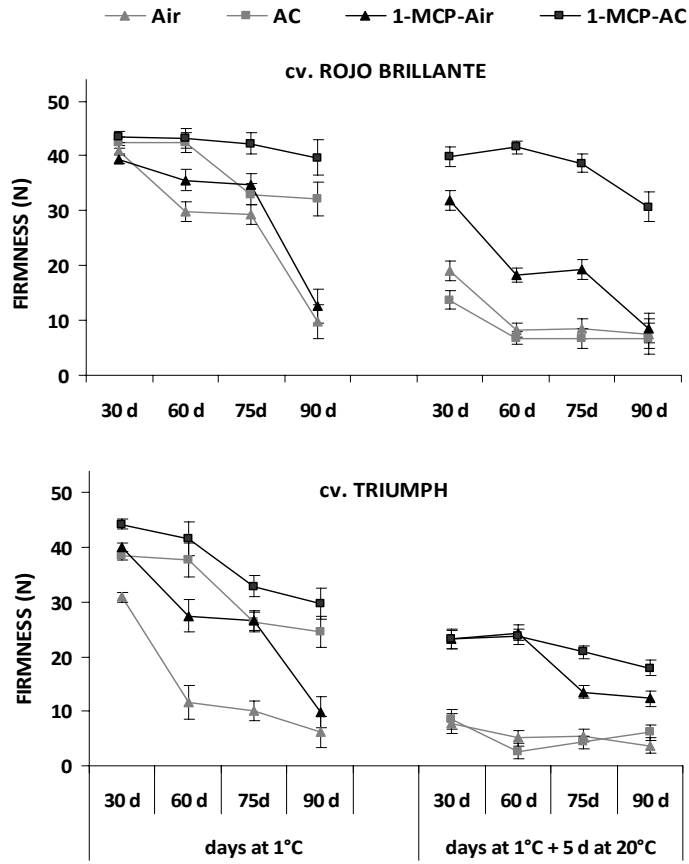


Fig. 1. Firmness in Newtons of persimmon cvs. Rojo Brillante and Triumph during storage at 1°C and the subsequent shelf-life period of 5 d at 20°C. The conditions during storage were as follows: in air (CTL), in a controlled atmosphere (4-5% O₂ + N₂) (CA), in air after a pretreatment with 1-MCP (1-MCP-Air), in a CA after a pretreatment with 1-MCP (1-MCP-CA). Vertical bars represent LSD intervals ($P < 0.05$).

Table 1. Flesh gelling (from 0 = very firm fruit with little give, no gelling, to 4 = flesh soft, gelling of 70-100% of cut surface) of persimmon cv. Rojo Brillante and cv. Triumph during storage at 1° C and a subsequent shelf-life period of 5 d at 20 °C.

		Days at 1°C				Days at 1°C + 5 days at 20°C			
<u>Treatment</u>		30 d	60 d	75 d	90 d	30 d	60 d	75 d	90 d
R.Brillante	Air ^a	0a ^c	1b	1c	1.8c	0.8b	2.3c	2.8c	2.8c
	CA ^b	0a	0a	0a	0.3a	0a	0.8b	1b	1.6b
	1-MCP-Air ^c	0a	0.3a	0.3b	1.0b	0a	0.8b	1b	1.1b
	1-MCP-CA ^d	0a	0a	0a	0a	0a	0a	0a	0a
Triumph	Air	0a	1.4b	1.6c	2.3c	1b	4c	4d	4c
	CA	0a	0a	0.7b	0.5a	0a	1.5b	2.7c	2.9b
	1-MCP-Air	0a	0.8b	1.1bc	1.3b	0a	1.2b	1.9b	2.3b
	1-MCP-CA	0a	0a	0a	0.3a	0a	0.2a	0.3a	0.5a

^a Storage in air (CTL)

^b Storage in a controlled atmosphere (4-5% O₂ + N₂)

^c Storage in air after a pretreatment with 1-MCP

^d Storage in a controlled atmosphere (4-5% O₂ + N₂) after a pretreatment with 1-MCP

For each cultivar, different letters in the same column indicates statistical differences $P < 0.05$ (LSD test)

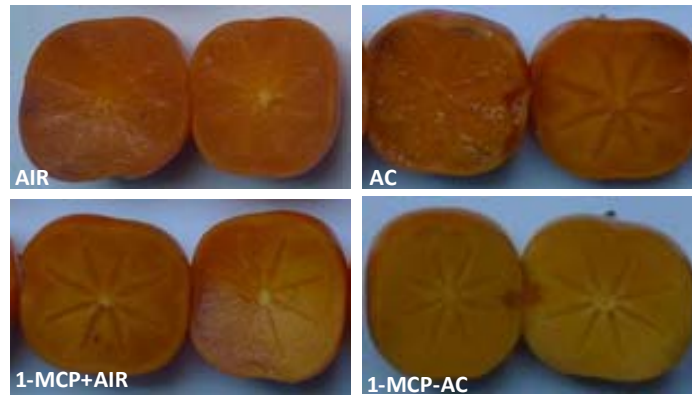


Fig. 2. Internal appearance of persimmon cv. Triumph stored for 75 days at 1°C plus a shelf-life period of 5 d at 20°C. The conditions during storage were as follows: in air (CTL), in a controlled atmosphere (4-5% O₂ + N₂) (CA), in air after a pretreatment with 1-MCP (1-MCP-Air), in a CA after a pretreatment with 1-MCP (1-MCP-CA).

3.2. External colour

‘Rojo Brillante’ and ‘Triumph’ fruits were harvested with an external colour index of +13. Fruits from both cultivars underwent a gradual increase in colour during the storage and subsequent shelf-life (data not shown). In the ‘Rojo Brillante’ fruit, no remarkable differences were observed among treatments; the colour index of all the fruits was between +18 and +24 after a 90-day conservation plus shelf-life. In ‘Triumph’, a greater colour index increase during low-temperature conservation was observed in the control fruit. However, after the different shelf-life periods, the external colour of the fruits was similar for all the treatments. Therefore, after the shelf-life that followed the 90-day storage, all the fruits showed colour index values between +20 and +25.

3.3 Soluble tannins and sensory evaluation of astringency

Both cultivars under study belong to the astringent group and are, therefore, characterised by having high soluble tannins (ST) content at harvest. 'Rojo Brillante' and 'Triumph' fruits had 0.5% and 0.9% ST, respectively, at harvest.

After 30 days of cold storage, the ST content of 'Rojo Brillante' fruits remained high (0.45-0.5%) in all the treatments (Fig. 3). For cold storage of between 30 and 60 days, ST content slightly decreased to values of around 0.4%, which then remained constant in all the treatments, except in the control fruit, in which ST declined to around 0.3% after 90 d. Orihuel et al. (2010) reported that during ULO cold storage, the soluble tannins of 'Rojo Brillante' fruits declined from 0.7 to 0.4% within 24 d. It must be noted that for 'Rojo Brillante' persimmons, the usual values for non-astringent fruit are 0.03-0.04% of ST. An ST concentration higher than 0.04%, and even one as low as 0.06%, may cause astringency (Besada et al., 2010b). In fact, the sensory evaluation of the fruit in the present study revealed that 'Rojo Brillante' fruits remained highly astringent (astringency value = 4) throughout cold storage. A similar pattern was observed in 'Triumph', which underwent a gradual, but slight, decline of soluble tannins during a 60-day storage at low temperature, with no notable differences found among treatments (Fig. 3). The ST content remained invariable in all the fruits until 90 days of storage. As in 'Rojo Brillante', panellists detected a high level of astringency throughout cold storage of 'Triumph' fruits (data not shown).

For both cultivars, and irrespectively of the storage conditions that fruits were subjected to, the deastringency treatment applied before the shelf-life period led to the drastic drop in ST content to values of 0.03% (Fig 3). It is known that the effectiveness of CO₂ treatment to remove astringency is based on insolubilization of tannins by the intermediation of the acetaldehyde generated during anaerobic respiration, which is triggered while fruits are exposed to a high CO₂ atmosphere (Matsuo and Itoo, 1982). In this study, the sensory test revealed that all the 'Rojo Brillante' and 'Triumph' fruits were evaluated as being free of astringency after the shelf-life period (sensory astringency value = 1). Therefore, any of the assayed storage conditions affected the effectiveness of the deastringency treatment with CO₂.

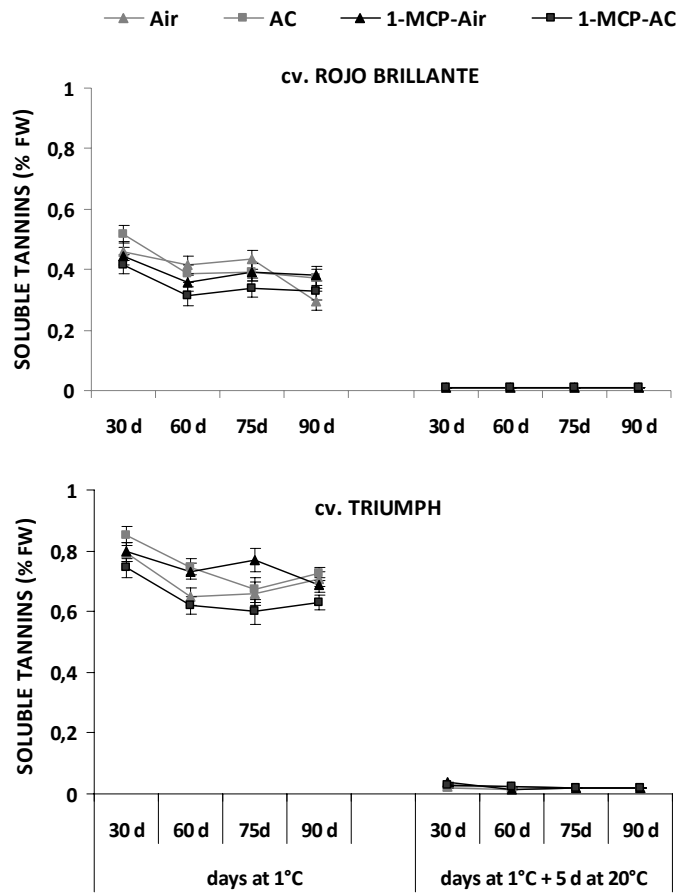


Fig. 3. The soluble tannins content of persimmon cvs. Rojo Brillante and Triumph during storage at 1°C and a subsequent shelf-life period of 5 d at 20°C. The conditions during storage were as follows: in air (CTL), in a controlled atmosphere (4-5% O₂ + N₂) (CA), in air after a pretreatment with 1-MCP (1-MCP-Air), in a CA after a pretreatment with 1-MCP (1-MCP-CA). Vertical bars represent LSD intervals ($P < 0.05$).

3.4. Acetaldehyde and ethanol concentration and sensory evaluation of off-flavours

In 'Rojo Brillante' fruit, the acetaldehyde concentration remained at similar levels to those at harvest (0.11 mg/100mL) during the first 30 days of cold storage (Fig. 4A). However, this volatile somewhat accumulated in all the treatments after being stored for 60 days at 1°C. Thereafter, acetaldehyde was gradually accumulated in the control fruit, while it remained unchanged in the remaining treatments. Thus, the control fruit showed an acetaldehyde concentration of 1 mg/100mL after 90 d at 1°C, while fruit acetaldehyde was lower than 0.6mg/100mL in the other treatments. In all the treatments, ethanol remained at very low levels throughout cold storage (Fig. 3B). Application of the CO₂ treatment before shelf-life periods triggered the production of both volatiles (Fig. 4A and 4B). Irrespectively of the storage treatment, fruits accumulated acetaldehyde to values higher than 2 mg/100mL, with ethanol values close to 50 mg/100mL.

In 'Triumph' fruit, acetaldehyde increased from 0.18 mg/100mL at harvest to reach values of 0.5 after 30 d of cold storage (Fig. 4A). Thereafter, a gradual increase in the acetaldehyde concentration, to reach values of 3 mg/100mL, was observed in the control and 1-MCP-treated fruits. In CA and the 1-MCP-CA treated fruits, acetaldehyde remained unchanged from 30 d to 90 d of storage. As observed in 'Rojo Brillante', ethanol accumulation was not detected during the cold storage period for any assayed treatment in 'Triumph' (Fig. 4B). In 'Triumph', the application of a deastringency treatment resulted in increased acetaldehyde and a higher level of ethanol than that observed in 'Rojo Brillante' (Fig. 4A and 4B). Moreover in 'Triumph', some remarkable differences were found between the control and the other treatments. Thus while the application of CO₂ treatment to the control fruit resulted in an increasing accumulation of volatiles during the shelf-life that followed storage of 30 d, 60 d and 75 d, the accumulation of these volatiles was similar in the rest of the treatments, irrespectively of the previous storage period. The most marked differences among treatments were observed after 75 d + shelf-life; the control fruit showed 12 mg/100mL and 200 mg/100mL of acetaldehyde and ethanol, respectively, while acetaldehyde was lower than 9 mg/100mL and ethanol concentration was between 100-120 mg/100mL in all the other treatments.

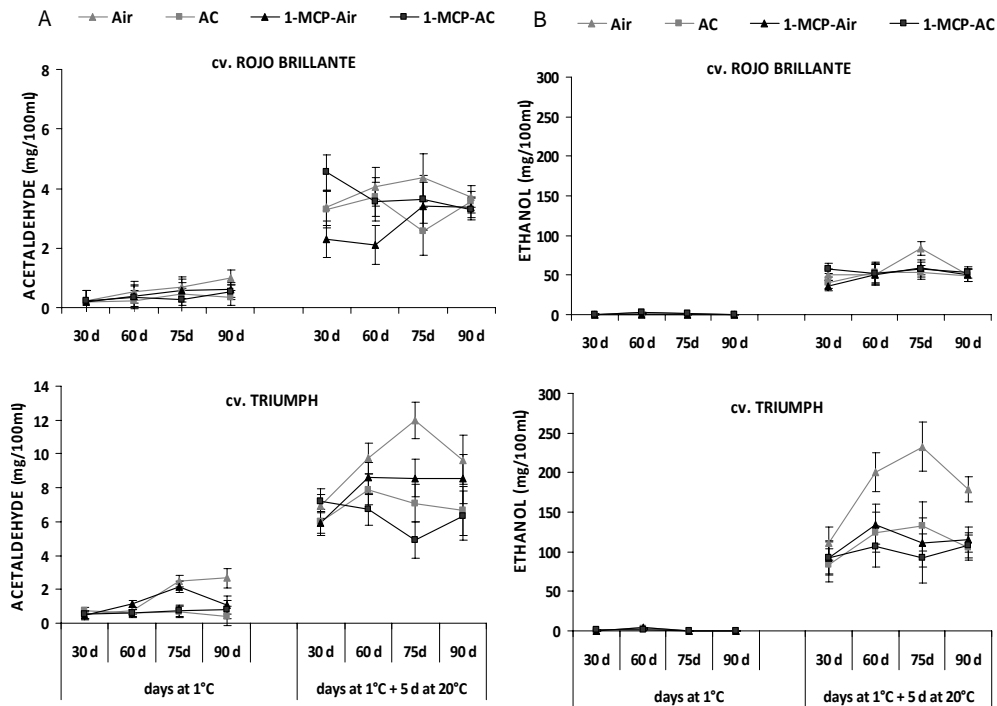


Fig. 4. Acetaldehyde (A) and ethanol content (B) (mg/100mL) of persimmon cvs. Rojo Brillante and Triumph during storage at 1°C and a subsequent shelf-life period of 5 d at 20°C. The conditions during storage were as follows: in air (CTL), in a controlled atmosphere (4-5% O₂ + N₂) (CA), in air after a pretreatment with 1-MCP (1-MCP-Air), in a CA after a pretreatment with 1-MCP (1-MCP-CA). Vertical bars represent LSD intervals ($P < 0.05$).

Arnal and Del Río (2004b) linked the CI of persimmon to increased levels of volatiles. Accordingly, the control fruits of both ‘Rojo Brillante’ and ‘Triumph’ in the present study were those with the highest levels of volatiles during cold storage and the subsequent shelf-life. The relation between CI and accumulation of volatile compounds was especially clear in the cv. Triumph because, during storage at 1°C, the level of acetaldehyde markedly increased in not only the control, but also in the 1-MCP-treated fruits, these being two treatments that showed the most striking flesh gelling (Fig. 4A and Table 1). In addition, during the shelf-life periods, the control fruits showed the greatest

acetaldehyde accumulation, which must be due not only to the anaerobic conditions during the deastringency treatment (Pesis et al., 1988), but also to CI severity (Arnal and del Río, 2004b).

Throughout the present study, no off-flavours were detected by the panellists in either 'Rojo Brillante' or 'Triumph' fruit (data not shown). Ke et al. (1991) reported that changes in the concentration of volatiles during storage might influence persimmon flavour. In persimmon, taste deterioration has been related to ethanol accumulation at levels exceeding 75 mg/100 mL (Ben-Arie et al., 1991). Nevertheless, the results of this study indicate that neither 100 mg/100 mL of ethanol (maximum ethanol production in 'Rojo Brillante') nor 200 mg/100 mL (maximum ethanol production in 'Triumph') had a negative effect on fruit flavour.

3.5. Internal Browning associated to CA

The internal fruit evaluation revealed the occurrence of internal browning associated with storage under CA conditions in 'Rojo Brillante' persimmons. In 'Triumph', this disorder was not observed.

So 'Rojo Brillante' fruits which were cold-stored in a CA, either with or without 1-MCP pretreatment (CA and 1-MCP-CA), manifested internal browning in the central fruit area, which was accompanied by changes in flesh consistence, (Table 2 and Fig. 5).

During storage at 1°C, internal browning was manifested from 75 days onwards. Nevertheless, under shelf-life conditions, this disorder was detected after 60 days of storage. Indeed, the longer the cold storage period, the more evident the damage after the shelf-life periods. Flesh browning is one of the main disorders associated with storage at low O₂ levels in persimmon fruit (Burmeister et al., 1997; Park and Lee, 2008). Ben-Arie et al. (1991) reported that accumulation of volatiles in a modified storage atmosphere of persimmon is a limiting factor that causes off-flavours and brown discoloration of peel and flesh. Park and Lee (2008) described different types of browning associated with a modified atmosphere in persimmon. These authors reported that while the incidence of style end browning was related with ethanol accumulation, other types of browning were not linked to the increment in this volatile. The CA-related flesh browning observed herein did not show any relation with the ethanol concentration in fruit. Thus, in 'Rojo Brillante', no relevant differences in ethanol or acetaldehyde concentration were observed between the CA-stored

fruit, which manifested flesh disorder, and air-stored fruit, in which flesh browning was not detected. Moreover, no internal browning was detected either during storage or after the shelf-life periods in ‘Triumph’ fruits, which generally accumulated a higher volatile concentration than ‘Rojo Brillante’ fruits. Lee et al. (2003) suggested that CA-induced flesh browning in persimmon is due mainly to a low-oxygen atmosphere, while high CO₂ seems to be a determinant for external injuries. Our results revealed that tolerance to a low-oxygen atmosphere was different in the ‘Rojo Brillante’ and ‘Triumph’ fruits, which corroborates the importance of investigating an adequate atmosphere for each cultivar.

Table 2. Internal browning (from 0 = absence, to 3 = severe) of persimmon cv. Rojo Brillante during storage at 1°C and a subsequent shelf-life period of 5 d at 20°C.

	Treatment	Days at 1°C				Days at 1°C + 5 days at 20°C			
		30 d	60 d	75 d	90 d	30 d	60 d	75 d	90 d
R.Brillante	Air ^a	0a ^c	0a	0a	0a	0a	0a	0a	0a
	CA ^b	0a	0a	0.5b	0.4b	0a	1.2b	1.5b	1.7b
	1-MCP-Air ^c	0a	0a	0a	0a	0a	0a	0a	0a
	1-MCP-CA ^d	0a	0a	0.5b	0.8c	0a	1.3b	1.5b	1.9b

^a Storage in air (CTL)

^b Storage in a controlled atmosphere (4-5% O₂ + N₂)

^c Storage in air after a pretreatment with 1-MCP

^d Storage in a controlled atmosphere (4-5% O₂ + N₂) after a pretreatment with 1-MCP

Different letters in the same column indicates statistical differences $P < 0.05$ (LSD test)



Fig. 5. Internal browning observed in persimmon cv. Rojo Brillante treated with 1-MCP and then stored in a CA (4-5% O₂ + N₂) for 75 days at 1°C plus a shelf-life period of 5 d at 20°C.

3.6. Decay incidence

Black spot caused by *Alternaria alternata* has been reported as the main postharvest factor that impairs the quality and reduces the storability of ‘Triumph’ persimmons (Kobiler et al., 2011). Similarly, *A. alternata* has been described as one of the main causal agents of latent and wound infections in ‘Rojo Brillante’ (Palou et al., 2009). This fungus infects fruits in the orchard and remains quiescent until harvest. After harvest, the pathogen slowly colonises the fruit during low-temperature storage (Kobiler et al., 2011).

In the present study, decay incidence was evaluated after a 90-day storage and a subsequent shelf-life period (Table 3). Our results revealed that ‘Triumph’ fruit showed greater susceptibility to *Alternaria alternata* than ‘Rojo Brillante’ fruit. So, the air-stored ‘Triumph’ fruits exhibited a decay index of 2.6 after 90 d of storage, which was 2.05 in the ‘Rojo Brillante’ fruits. In this study, no relevant effect of 1-MCP by itself was observed on fruit decay, which is in accordance with that previously reported for cv. Quioto (Brackmann et al., 2003) and cv. Fuyu (Vilela et al., 2007; Krammes et al., 2006).

The fruits from both cultivars stored under CA conditions, and pretreated or not with 1-MCP (CA and 1-MCP-CA), showed reduced decay incidence

when compared to the air-stored fruits. Hence, the CA fruits showed a decay index of 0.6-0.7 after 90d at 1°C for ‘Rojo Brillante’ and of 0.9 for ‘Triumph’. Prusky et al., (1997) reported that the atmosphere generated inside MA packs of persimmon stored for 4 months at low temperature significantly lowered the incidence of *A. alternata*. Low O₂ levels may exert a fungistatic effect by inhibiting spores germination and fungus development (Neuwald, 2004).

When fruit were transferred to the shelf-life conditions, the decay index sharply increased for all the treatments, but the effect of the CA treatment on lowering the decay index was still observed.

Table 3. Decay index (from 0 = absence, to 5 = severe) of persimmon cv. Rojo Brillante and cv. Triumph after 90 d of storage at 1°C and a subsequent shelf-life period of 5 d at 20°C.

	<u>Treatment</u>	90 d	+ 5d at 20°C
R.Brillante	Air ^a	2.05b ^c	2.87b
	CA ^b	0.74a	1.21a
	1-MCP-Air ^c	1.69b	2.05ab
	1-MCP-CA ^d	0.6a	1.1a
Triumph	Air	2.6b	3.87b
	CA	0.94a	1.32a
	1-MCP-Air	2.27b	3.03b
	1-MCP-CA	0.87a	1.08a

^a Storage in air (CTL)

^b Storage in a controlled atmosphere (4-5% O₂ + N₂)

^c Storage in air after a pretreatment with 1-MCP

^d Storage in a controlled atmosphere (4-5% O₂ + N₂) after a pretreatment with 1-MCP
Different letters in the same column indicates statistical differences $P < 0.05$ (LSD test)

4. Conclusion

Our results revealed that ‘Triumph’ cultivated in Spain, besides the important firmness loss previously reported in post-cold storage shelf-life, develops flesh gelling which is a evident chilling injury symptom. This disorder was more severe in ‘Triumph’ than in ‘Rojo Brillante’, in which the main CI symptom is drastic softening when fruit are transferred from low to shelf-life temperatures.

The CA based on 4-5% O₂ + N₂ did not have any additional benefit on retarding softening in relation to the 1-MCP pre-treatment in both cultivars. In ‘Rojo Brillante’, the CA storage resulted in internal browning from 60 days of storage onward. However, the combined use of the 1-MCP pretreatment and CA storage prolonged the storage of ‘Triumph’ to 90 days by alleviating both fruit softening and flesh gelling. In ‘Rojo Brillante’, this treatment also significantly retarded fruit softening and inhibited gelling development, but the occurrence of internal browning became the most important limiting factor. The use of this CA clearly has an effect on retarding decay development.

Therefore, the CA (4-5% O₂ + N₂) storage of fruits previously treated with 1-MCP is shown to be a useful technology to prolong storage of ‘Triumph’ as it helps preserve external and internal fruit quality. Nevertheless, in ‘Rojo Brillante’, CA results in severe internal damage, which impairs fruit quality. Thus, appropriate CA storage conditions must be optimised for this cultivar.

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CHAPTER VI

Short-term high CO₂ treatment alleviates chilling injury of persimmon cv. Fuyu by preserving the parenchyma structure

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Abstract

Persimmon cv. Fuyu is chilling-sensitive and manifests chilling injury symptoms, flesh gelling and fruit darkening, during cold storage and subsequent shelf-life. The objective of this study was to evaluate the effect of short-term high CO₂ treatments on chilling injury manifestation of 'Fuyu'. Short-term high CO₂ treatments consisted in maintaining fruit in a 95% CO₂ atmosphere for 0, 12, 24 or 36 h before storage at 1°C. After 35d and 50d of low-temperature storage and after subsequent shelf-life periods of 5d at 20°C, fruit quality and microstructural changes of flesh were evaluated. Our results showed that short-term high CO₂ treatments alleviate the main chilling injury symptoms, flesh gelling and fruit darkening. The longer the treatment, the greater chilling injury alleviation becomes. The microstructural study revealed that flesh gelling is associated with a complete disruption of cell walls and membranes, which led to the total loss of the initial parenchyma structure. Short-term high CO₂ treatment alleviated flesh gelling by preserving the integrity of cells walls and plasmalemma.

Keywords: Persimmon, Short-term high CO₂ treatment, Chilling injury, Flesh gelling, Microstructure

1. Introduction

Persimmon fruit (*Diospyros kaki* L.), as many tropical and subtropical fruits, are sensitive to low temperature. The sensitivity of persimmon to chilling injury is cultivar-dependent; cultivars such as ‘Fuyu’, ‘Sugura’ or ‘Rojo Brillante’ are very chilling-sensitive, whereas others, such as ‘Triumph’ or ‘Hachiya,’ are not so susceptible to this disorder (Collins & Tisdell, 1995). Although chilling injury symptoms can vary depending on the cultivar, flesh texture disorders are reported in all sensitive cultivars as one of the main chilling injury manifestations. In ‘Fuyu’, which is a commercially important non-astringent persimmon in several countries, including Japan, Brazil, Korea, Australia and New Zealand, chilling injury is expressed initially in the form of a gel developing within flesh, and later by fruit darkening and increased skin transparency through which the characteristic gel can be seen (MacRae, 1987a). During not overly long storage periods, chilling injury symptoms usually appear when transferring fruits to shelf-life temperatures. However during prolonged storage, such symptoms can eventually appear during cold storage (MacRae, 1987b). Since ‘Fuyu’ is free of astringency at harvest and does not require deastringency treatment, fruits are generally consumed “crisp”. Therefore preserving fruit texture is the main challenge when storing fruits (Park & Lee, 2005).

Short-term anoxic treatment has been reported to alleviate the flesh firmness disorder associated with chilling damage in fruits such as avocado (Pesis, Marinansky, Zauberman & Fuchs, 1993), kiwi (Song, Gao, Chen, Mao, Zhou, Chen & Jiang, 2009) or peach (Polenta, Budde & Murray, 2005). Besides, other chilling symptoms such as browning of litchi (Liu, Song, Jiang, Joyce, Zhao, You & Wang, 2007) and avocado (Pesis et al., 1993), rind pitting of grapefruit (Hatton, Cubbedge & Grierson, 1975), and irregular ripening of tomato peel (Ibrahim, Rhani & Buhri, 2013) are alleviated by short-term anoxic treatments.

Several studies have suggested that the effect of postharvest disorder alleviation exerted by anoxic treatments might be related to their effect on the redox system, and finally on the cellular membrane. Thus in fruits such as kiwi, loquat or litchi, which were treated with short-term anoxia, enhanced activity of ROS scavenger enzymes (SOD, CAT or APX) and membrane integrity preservation have been associated with chilling injury alleviation (Liu et al., 2007; Song et al., 2009; Gao, Tao, Song, Chen, Chen, Zhou, Mao & Zheng, 2009).

In the persimmon cultivars belonging to the astringent group, short-term anoxic treatments based on high CO₂ concentration (80-95%) applied for 12-24h have been widely studied because of their astringency removal effect (Besada, Arnal, Salvador & Martínez-Jávega, 2010; Novillo, Besada, Gil & Salvador, 2013). Among the numerous studies that have addressed effectiveness of CO₂ treatment as a deastringency method, several have involved low-temperature fruit storage after astringency removal, however no effect of chilling damage alleviation has been observed. Recently marked changes in the redox system consequence of CO₂ treatment have been described in astringent persimmon 'Rojo Brillante', where the activity of the CAT, APX and SOD scavenging enzymes was up-regulated after deastringency treatment (Novillo, Salvador, Magalhaes & Besada, 2014). It is known that the effects of anoxic treatments greatly depend on commodity type, but also on the intensity of the application (Fallik, Polevaya, Tuvia-Alkalai, Shalom & Zuckermann, 2003; Polenta et al., 2005). Therefore, it would be of interest to study the response of non-astringent persimmons to short-term high CO₂ treatments which have been never approached. Despite the link between the beneficial effects of short-term anoxic treatments with membrane preservation being reported in the aforementioned studies, to our knowledge this fact has not yet been confirmed by microscopy techniques.

Therefore, the aim of this article was to study the effect of short-term high CO₂ treatments (12, 24, 36 h) on the quality of non-astringent persimmon 'Fuyu' after prolonged low-temperature storage. Besides, the relationship between the effects that short-term high CO₂ treatments have on fruit quality and the microstructural changes of flesh were evaluated during storage and shelf-life periods.

2. Materials and Methods

2.1. Plant material and treatments

Persimmon (*Diospyros kaki* L.) cv. Fuyu, introduced from Brazil and grown in Spain (8-year-old trees), were harvested in l'Alcúdia (E Spain) at commercial maturity stage when fruits presented an external colour index = 11.6, a firmness value of 54 N and total soluble solids of 15.3°Brix. After harvest, fruits were taken to the Instituto Valenciano de Investigaciones

Agrarias (IVIA), where they were carefully selected for uniformity of size and colour, and for lack of defects. Twenty lots of 20 fruits were formed to be exposed to CO₂ treatment in closed containers (95% CO₂ at 20°C and 90% RH) for 0, 12, 24 and 36 h (five lots of 20 fruit per time). After each CO₂ treatment, one lot of fruit was maintained for 1 day at 15°C and was then analysed. The remaining lots of fruit were stored at 1°C for up to 50 days. After 35 and 50 days of storage, two samples of 20 fruits per treatment were removed from the cold storage room. One sample was analysed directly and the other was transferred to 20°C for 5 d to simulate the shelf-life period.

2.2. Fruit assessments

After CO₂ treatment, the following analyses were carried out: flesh firmness, external colour, soluble tannin content, acetaldehyde and ethanol concentration, and ethylene and CO₂ production rate. After 35 and 50 days of storage and the subsequent shelf-life periods, fruit firmness, external colour, and the incidence and severity of chilling injury, were all evaluated. Sensory evaluation and microstructural studies by Scanning Electron Microscopy at low temperature (Cryo-SEM) were carried out on flesh after CO₂ treatment, and the cold storage and shelf-life periods. Besides, ethylene and CO₂ production were evaluated daily throughout the shelf-life periods.

2.2.1. Physiological measurements

Skin colour was evaluated on the samples of 20 fruits by measuring 'L' and 'b' Hunter parameters using a Minolta Colorimeter (Model CR-300, Ramsey, NY, USA).

Chilling injury was evaluated by incidence (percentage of fruit manifesting flesh gelling) and severity (scale from 0 to 4) by methods modified from Woolf, Ball, Spooner, Lay, Ferguson, Watkins, Gunson and Forbes (1997). CI severity was rated on a 5-point scale according to the amount of gelling detected in the flesh of fruits cut through the equator: 0 = fruit with little give, no gelling; 1 = flesh with slight-moderate give, no gelling, feels slightly slippery to the touch; 2 = flesh with moderate give, slight gelling (10-30% of the cut surface), feels slippery to the touch; 3 = flesh generally soft, gelling (darker orange) of 30-70% of cut surface, flesh slippery to touch; 4 = flesh soft, gelling (darker orange) of 70-100% of cut surface, flesh slippery to touch. The CI data are presented as a percent of incidence (proportion of fruit with rating >0) or as average severity.

Flesh firmness was evaluated in a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA, USA) using an 8-mm plunger. Fruit firmness values were an average of 18 fruits per treatment. The results are expressed as load in Newtons (N) to break flesh in each fruit on 180° sides after removing peel.

To determine soluble tannins (ST) and acetaldehyde and ethanol concentration, lots of 15 fruits per treatment were divided into three samples and cut into four longitudinal parts. One part was sliced and frozen at -20°C to determine ST. Two other opposite fruit parts were placed in an electric juice extractor and filtered juice was then used to determine acetaldehyde and ethanol concentration. The remaining fruit part was destined to sensory evaluation. ST were evaluated by the Folin-Denis method (Taira, 1995); the results were expressed as a percentage of fresh weight. Three replicates of acetaldehyde and ethanol concentration were measured per juice sample and analysed by headspace gas chromatography as described by Salvador, Arnal, Monterde and Cuquerella (2004).

A 4-point scale was used for sensory evaluation of the fruit flavour, from 0=absence of off-flavours to 3=intense off-flavours. Each sample consisted of segments which were taken from about five individual fruits. Samples were presented to the panellists in trays labelled with 3-digit random codes and were served at room temperature. The panellists had to taste several segments of each sample in order to compensate, as far as possible, for the biological variation of the material. Milk was provided for palate rinsing between samples.

Carbon dioxide and ethylene production were determined daily during the shelf-life period at 20°C . Three fruits were weighed and individually sealed in 1-L glass jars for 2 h at 20°C and then 1 mL of the headspace sample was injected into a Perkin Elmer gas chromatograph equipped with TCD and FID detector as described by Salvador, Arnal, Monterde and Martínez-Jávega (2005). CO_2 production was expressed as $\text{mmol CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ and ethylene production as $\text{nmol C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$.

The data were subjected to analysis of variance, and multiple comparisons between means were determined for each period of evaluation by the least significant difference test ($p = 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA).

2.2.2. Cryoscanning Electron Microscopy (Cryo-SEM)

Cubes (3mm³) were cut from the equatorial area perpendicularly to the main axis of the persimmon flesh with a stainless steel cutter. These cubes were then immersed in slush nitrogen (−210°C) and were then transferred to a cryo-trans (CT 15000 C from Oxford Instruments, Oxford, England) linked to a scanning electron microscope model JEOL JSM5410 (JEOL, Tokyo, Japan), operating at a temperature below −130°C. Samples were cryofractured at −180°C, etched at −90°C and coated with gold. Observations under the microscope were made at 15 kV and at a working distance of 15 mm.

3. Results

3.1. Flesh gelling

After 35 days of storage, control fruit showed a high incidence of flesh gelling, 85%, which increased to 100% after 50 days (Fig. 1A). Average flesh gelling severity was 1.6 and 2.2 after 35 and 50 days at 1°C, respectively (Fig. 1B). The shelf-life conditions severely aggravated chilling injury manifestation. Thus, fruits were chilling injured with high severity, 3.8, after the shelf-life periods that followed 35 and 50 storage days. The short-term high CO₂ treatments applied before cold storage greatly reduced CI incidence and severity during the low-temperature storage and shelf-life periods. The longer the treatment duration, the greater CI alleviation became. Thus after 50 storage days at 1°C, slight chilling injury (severity<1) was manifested in around 40% of the fruits treated for 12 h and 24 h, while this disorder was not observed in the 36-hour-treated fruit. As in the control fruits, the incidence and severity of damage increased when fruits were transferred to moderate temperatures. After the shelf-life that followed 50 storage days, 60% of the fruits treated for 12 h and 24 h were chilling-affected (1.3-1.5 severity) and 30% of the 36-hour-treated fruit showed very slight flesh gelling (CI severity of 0.4).

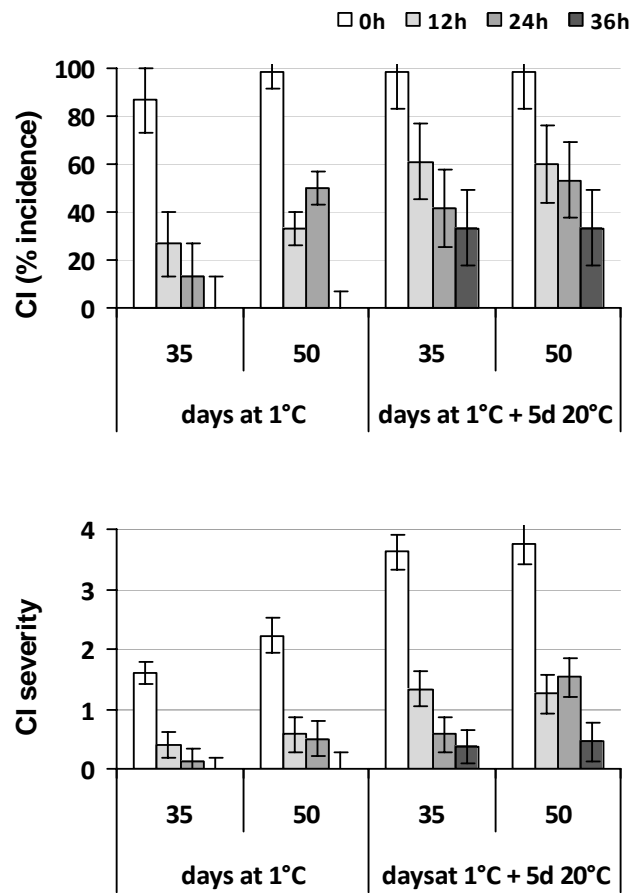


Fig. 1. Effect of short-term high CO₂ treatments (12-36 h of CO₂ 95%) on incidence (% of fruit affected) and severity (from 0 to 4) of chilling injury in 'Fuyu' persimmons stored for 35 and 50d at 1°C, plus shelf-life periods of 5d at 20°C. Vertical bars represent the LSD intervals ($p < 0.05$).

3.2. Firmness

Fruit showed firmness values of 54N at harvest time. Throughout the storage period, the control fruits underwent gradual softening to 43N and 23N after 35 and 50 days at 1°C, respectively (Fig. 2). Short-term high CO₂ treatments alleviated fruit softening during low-temperature storage. After 35 days at 1°C, this effect was observed only in the 36-hour-treated fruit. However after 50 days of storage, all the CO₂-treated fruit presented higher firmness (close to 40N) than the control fruits (23N).

Although when fruits were transferred to shelf-life conditions a drastic loss of firmness to values lower than 10N was observed in all the treatments, the lowest firmness values were detected in control fruit.

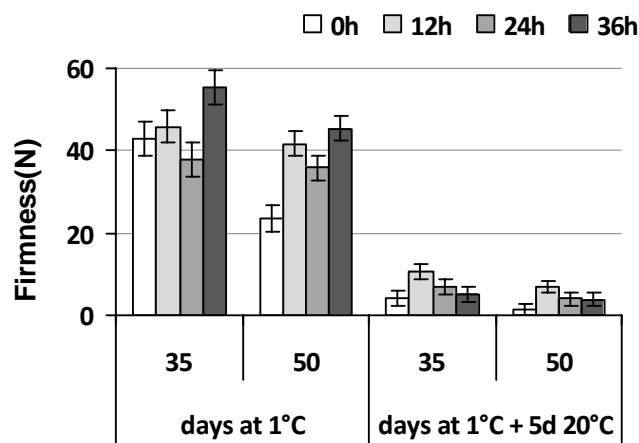


Fig. 2. Effect of short-term high CO₂ treatments (12-36 h of CO₂ 95%) on the firmness of 'Fuyu' persimmons stored for 35 and 50d at 1°C, plus shelf-life periods of 5d at 20°C. Vertical bars represent the LSD intervals ($p < 0.05$).

3.3. Skin Colour

At harvest time, fruits showed an L-value of 58 and a b-value of 33.8. As low-temperature storage advanced, both the colour parameters gradually lowered for the control fruits (Fig. 3A, 3B), which indicates gradual darkening and loss of yellowness. When fruits were transferred to shelf-life temperatures after 35 and 50 storage days, the L-value dropped to 39 and the b-value to around 14. Short-term CO₂ treatments reduced the decline of the L- and b-values in association with storage and shelf-life conditions. Thus changes in colour were mitigated by all the CO₂ treatments. This effect became more marked with longer exposure to CO₂. Thus the 36-hour treatment preserved external fruit colour similarly to that at harvest after the different storage periods and alleviated the colour changes after shelf-life. The 12-hour- and 24-hour-treated fruits showed intermediate L and b-values between the control and the 36-hour-treatment.

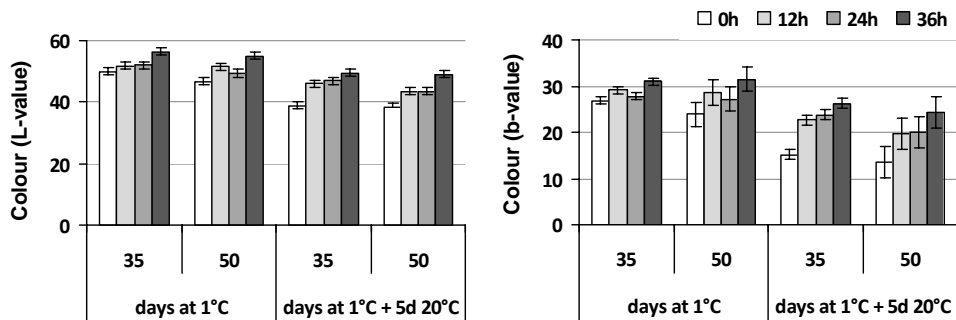


Fig. 3. Effect of short-term high CO₂ treatments (12-36 h of CO₂ 95%) on skin colour (L- and b-value) of chilling injury in 'Fuyu' persimmons stored for 35 and 50d at 1°C, plus shelf-life periods of 5d at 20°C. Vertical bars represent the LSD intervals ($p < 0.05$).

3.4. Ethylene and CO₂ production rate

The carbon dioxide and ethylene production rates of the fruits were determined after applying high CO₂ treatments (0 h, 12 h, 24 h and 36 h) and also daily throughout the shelf-life periods following storage. Fruits showed 6.7mg/kg.h CO₂ and undetectable levels of ethylene at harvest time (0-hour-treatment) (data not shown). After applying CO₂ treatments, an increase in the CO₂ rate was observed. The 12-hour- and 24-hour-treated fruits showed 17mg/kg.h CO₂, while the 36-hour-treatment led to higher CO₂ production, 29.3 mg/kg.h CO₂ (data not shown). The ethylene rate was not affected by high CO₂ treatments because the levels of ethylene remained undetectable after different CO₂ exposures (data not shown).

During the shelf-life period that followed 35 days storage, no significant differences were observed in the CO₂ rate among treatments. After removing fruits from cold storage plus 5 h at 20°C (d0), all the fruits obtained a respiration rate of 10-13 mg/kg.h. All of the treatments showed a slight peak of respiration after 1 day at 20°C and then returned to the values observed on day 0. During shelf-life, which followed 50 days of storage, different profiles were distinguished in the control and treated fruit. Whereas the respiration rate of high CO₂-treated fruit showed a gradual increase to reach values of 30 mg/kg.h after 3 days at 20°C, respiration in the control fruits increased from day 0 to day 1, and then displayed constant values at around 20 mg/kg.h.

Ethylene production (data not shown) was detected only in the 36-hour-treated fruit during the shelf-life that followed the 50-day storage. On day 0, the ethylene rate was 0.96 µg/kg.h, but then it lowered to values which came close to 0.30 µg/kg.h to increase again and peak at 1.26 µg/kg.h on day 4.

3.5. Soluble Tannins, Acetaldehyde and Ethanol concentration

Soluble tannins content, acetaldehyde and ethanol concentration were analysed at harvest time and after CO₂ treatments (after 1 day at 15°C) (Table 1). At harvest time, fruits showed 0.05% of ST, a low acetaldehyde concentration (0.51mg/100mL) and undetectable ethanol levels. Application of CO₂ treatments did not affect ST content but led fruit to volatile compounds accumulation. The concentrations of acetaldehyde and ethanol significantly increased the longer CO₂ exposure became. So after fruits were treated for 36 h,

the concentration of volatiles increased to 2.22 mg/100mL of AcH and 58.3 mg/100mL EtOH, while shorter treatments resulted in concentrations of acetaldehyde and ethanol of around 1.5 mg/100mL and 30-35 mg/100mL, respectively. Regarding sensory analyses, panellists reported the absence of fruit off-flavours irrespective of the treatment and evaluation period. Thus, all the fruit was rated with 0 (absence of off-flavours).

Table 1. Effect of short-term high CO₂ treatments (from 0-36 h of CO₂ 95%) on the contents of acetaldehyde (AcH), ethanol (EtOH) and soluble tannins of 'Fuyu' persimmons. Values presented are measurement replication means (n=3 replicates). Values with the same letter in each column were not significantly different according to LSD test ($p < 0.05$).

CO ₂ treatment duration	AcH (mg/100mL)	EtOH (mg/100mL)	S.Tannins (% fw)
0 h	0.51a	0.00a	0.05a
12 h	1.35b	30.61b	0.04a
24 h	1.59c	36.11c	0.04a
36 h	2.22d	58.32d	0.04a

3.6. Microstructure Changes

At harvest, the parenchyma of persimmon, observed by Low Temperature Scanning Electron Microscopy (Cryo-SEM), was compact with rounded, swollen cells and small air-filled intercellular spaces (Fig. 4A). Most cells were almost totally occupied by a large vacuole full of soluble material, observed as a network. This is the typical eutectic artefact generated in the water sublimation process while samples are prepared by Cryo-SEM (Neri et al, 2011). Some particular cells had a compact mass inside their vacuole, which indicates presence of insoluble compounds (Fig. 4B), which does not lead to the generation of the eutectic artefact in the sublimation process, observed by Cryo-SEM. These cells are called tannic cells (Yonemori, Oshida & Sugiura, 1997), and their vacuole contains insoluble tannins.

The parenchyma of the control fruits after 35 and 50 days at 1°C is shown in Figure 5. After 35d at 1°C, rounded parenchymatic cells with intercellular spaces among them were observed; some of these spaces appeared to be filled

with solutes, indicating cellular stress. At higher magnification, the cell walls of adjacent cells were detached from each other in some areas and soluble material was observed between them. After 50 d of cold storage, most intercellular spaces were occupied with soluble material and, in some areas, cellular integrity was lost and individual cells were hardly distinguished.

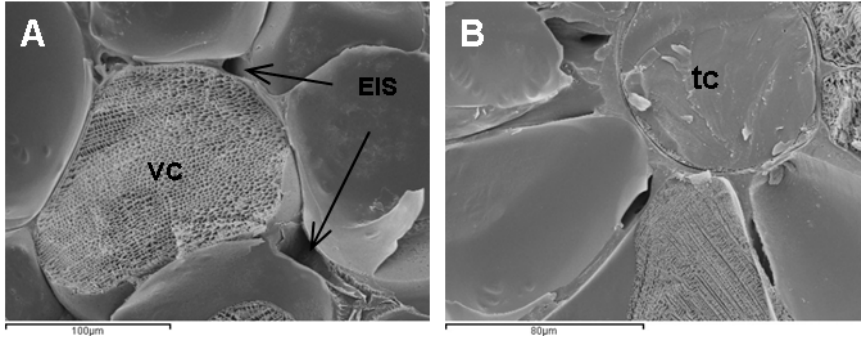


Fig. 4. Observation made by CryoSEM of the parenchyma structure of 'Fuyu' persimmons at harvest time. VC- vacuola filled with soluble material; tc-tannic cell; EIS- empty intercellular space.

The CO₂ treatments applied before storage for 12 hours (Fig. 5) gave way to diminished cellular degradation, both in the fruits stored for 35 and 50 days. Cell walls were better identified and the contours of the cells were well-defined. The separation between neighbouring cells was still observed, but whereas some intercellular spaces were filled with soluble material, others were empty. When CO₂ treatments were applied for 24 h and 36 h, the persimmon structure was preserved in the fruits stored for 35 and 50 days, and swollen cells whose cell walls were closely bonded to each other by intact cellular cements were observed. The plasmalemma also maintained its integrity mainly in the fruits stored for 36 h. Treatment seemed to reinforce cell walls and cellular cements, and preserved cell to-cell contact.

Transferring the control fruits to shelf-life conditions dramatically increased the cellular structure deterioration observed during low-temperature storage (Fig. 6). Cell walls and the plasmalemma were very thin and hardly observed. Therefore, the typical parenchymatic tissue was no longer observed as well-delimited independent cells surrounded by their cell walls, but a continuous mass of soluble material was observed, which could be related to the

gelling phenomenon associated with chilling injury. These microstructural changes which occurred under shelf-life conditions were more marked in the fruits stored for 50 days than in those stored for 35 days, which indicates greater tissue damage when fruits were stored longer. The cell walls and plasmalemma of the 12-hour-treated fruits (Fig. 6) were thin. At high magnification, the cell walls were detached from neighbouring cells and formed gaps filled with solutes. These solutes could be indicating the dissolution of the material from the cell walls and the middle lamella. However, cellular integrity was not completely lost as in the untreated fruits (0 h), where cell walls had almost totally degraded, which demonstrates that CO₂ treatment improves the fruit structure, even when short treatment times -12 h- are applied.

During the shelf-life that followed cold storage periods, the structure of the fruits treated for 24 h and 36 h was better preserved than in the 12-hour-treated fruits and the control persimmons. Cell walls and plasmalemmas maintained their integrity and were observed thicker than in the 0-h- and 12-hour-treated fruits. However, if these samples were compared to their counterparts before shelf-life, cellular cements remained intact and to firmly bond the neighbouring cells in fruits before being transferred to shelf-life (Fig. 5), while cells were detached from each other in the samples transferred to shelf-life and intercellular adhesion was lost (Fig. 6). Furthermore, some intercellular spaces also became filled with soluble material during shelf-life.

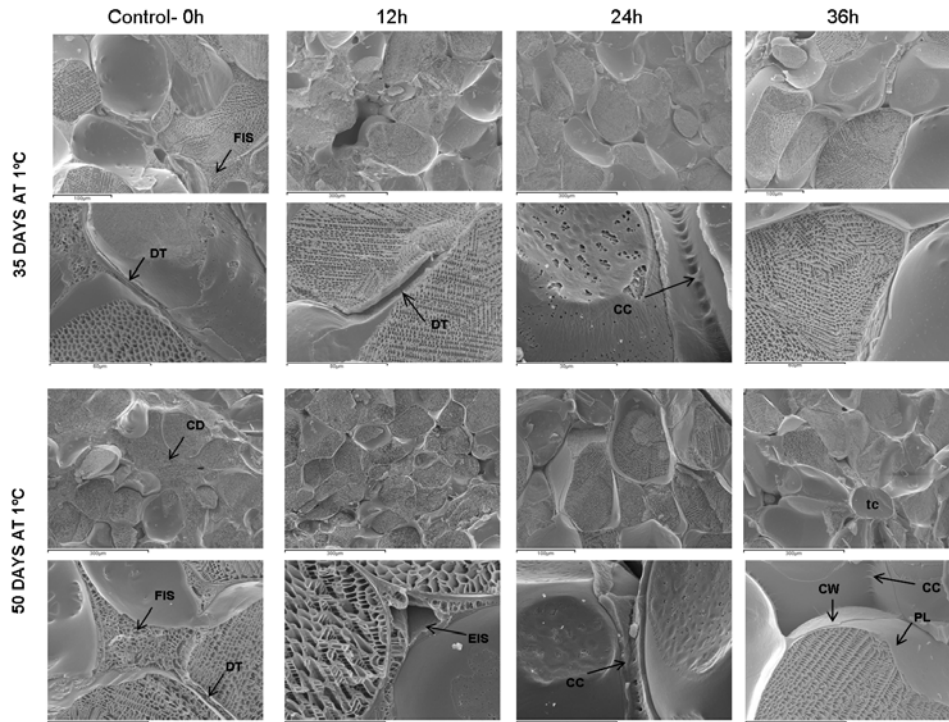


Fig. 5. Observation made by CryoSEM of the parenchyma structure of ‘Fuyu’ persimmons submitted to short-term high CO₂ treatments (from 0-36 h of CO₂ 95%) and stored for 35 and 50 days at 1°C. FIS- filled intercellular space; EIS- empty intercellular space; DT-cell wall detachment; CC-cement cellular; CD- cellular degradation; CW-cellular wall; PL-plasmalemma

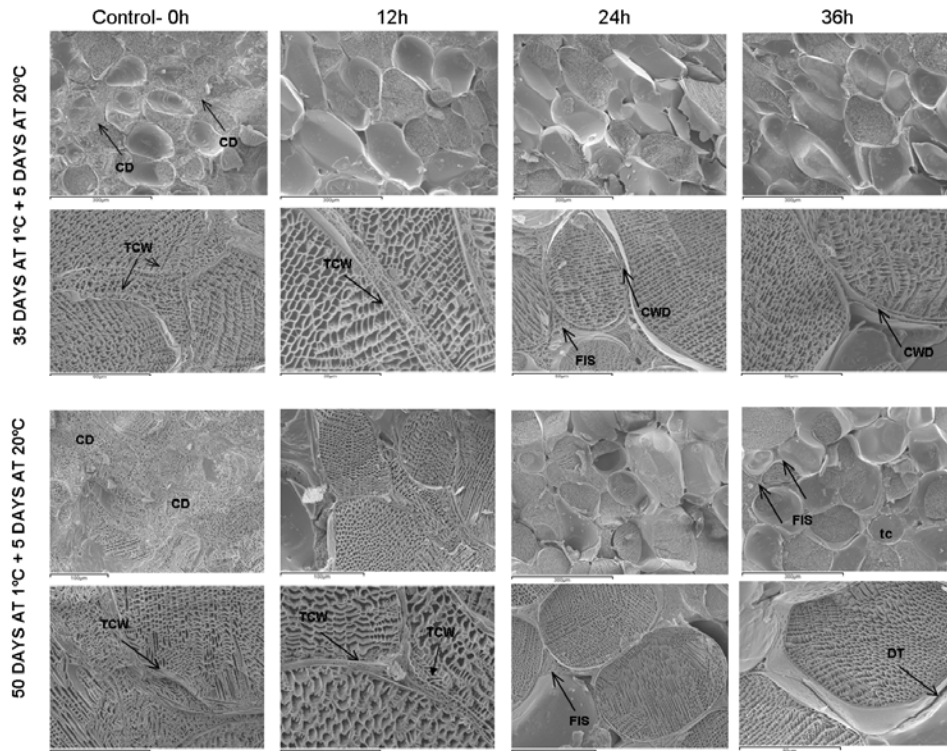


Fig. 6. Observation made by CryoSEM of the parenchyma structure of 'Fuyu' persimmons submitted to short-term high CO₂ treatments (from 0-36 h of CO₂ 95%) and stored for 35 and 50 days at 1°C, plus shelf-life periods of 5d at 20°C. CD- cellular degradation; TCW-thin cell walls; DT-cell wall detachment ; CWD-cell wall degradation; FIS- filled intercellular space; tc-tannic cell

4. Discussion

In 'Fuyu' persimmon, chilling injury is expressed initially in the form of a gel that develops in flesh, and later by fruit darkening and skin transparency (MacRae, 1987a). In the present study, both symptoms (flesh gelling and fruit darkening) were manifested in the control fruits after the studied cold storage periods and were aggravated after the subsequent shelf-life.

Short-term high CO₂ treatments significantly reduced the incidence and severity of chilling injury. The longer the high CO₂ treatment, the more alleviated chilling injury symptoms become. So during the shelf-life periods following cold storage, the fruits treated for 36 h showed the lowest incidence and flesh gelling severity and obtained the highest L- and b-values.

In the control fruits, in parallel to flesh gelling development, reduced firmness was exhibited. In the short-term high CO₂-treated fruits, flesh softening was also observed until similar firmness values were obtained to those of the controls, but loss of firmness was not related to flesh gelling symptoms. The microstructural study of fruit flesh during cold storage and the subsequent shelf-life revealed some major differences in the changes taking place in the parenchyma structure of the fruits affected by gelling when compared with that of the softened fruits with no gelling manifestation. So, in the control fruits, development of flesh gelling during storage is observed initially as a detachment of the cell walls between neighbouring cells and as a filling of intercellular spaces with solutes. The aggravation of gelling damage when fruits were transferred to shelf-life conditions is associated with the disruption of cell walls and plasmalemma, with complete loss of the delimited cell structure. In the fruits treated for 36 h, which exhibited softening with a very slight flesh gelling incidence, the original cellular integrity and intercellular cements were preserved during cold storage. When fruits were transferred to shelf-life conditions, marked fruit softening was associated with loss of intercellular cements and detachment of neighbouring cells, but cells were clearly delimited. In the fruits treated for 12 h and 24 h, in which flesh gelling was mildly alleviated, although cellular integrity was partly lost, most cells were still delimited. Therefore the microstructural study showed that the flesh gelling manifestation in chilling injured fruits would be associated with a severe disruption of cell walls and the plasmalemma with the subsequent cell content output to intercellular spaces. This confirms that the degree of solubilisation of cell walls in chilling-injured fruits is greater and that the process is more accelerated than in normal ripened fruit (Grant, MacRae and Redgwell, 1992).

Similarly, Zhang, Zhang, Huber, Rao, Sun and Li (2010) observed that the chilling injury of 'Fuyu' is associated with increased membrane permeability and malondialdehyde content, which are used as a direct indicator of membrane injury.

It is important to note that the differences in flesh gelling severity noted among treatments were not reflected in the measured firmness values. Therefore this parameter would be not an accurate indicator of neither flesh gelling nor of the changes undergone in the parenchyma of 'Fuyu'.

Persimmon fruits are considered climacteric fruit. However, ethylene production is usually very low (Salvador, Arnal, Besada, Larrea, Quiles & Pérez-Munuera, 2007); in fact in this study, its levels were undetectable at harvest time. Development of chilling injury symptoms has been associated with increased ethylene levels in different commodities, including persimmons (MacRae, 1987b; Park & Lee, 2005). Unexpectedly, our results showed that after storage periods studied, ethylene was undetectable in the control and also in the 12-h and 24-hour-treated fruits. Ethylene was detected only in the 36-hour-treated fruits after the shelf-life that followed the 50-day storage. Therefore it was the treatment that led to the greatest chilling injury alleviation which was the only one to stimulate ethylene production. Thus the ethylene production observed in the 36-hour-treated fruits probably contributes to the softening associated with normal fruit ripening. This is in accordance with Souza, Souza, Tiecher, Girardi, Nora, Silva, Argenta and Rombaldi (2011), who associated flesh gelling alleviation in 'Fuyu' by a prestorage acclimatisation treatment with the higher ethylene production of acclimatised fruits when compared to untreated ones which manifested flesh gelling symptoms. These authors also associated treated fruit softening with ethylene production and subsequent changes in the activity of endo-1,4-B-gluc, PME, PG and B-gal enzymes, while no activation of these enzymes was detected in the fruits manifesting flesh gelling; thus these authors attributed the breakdown of chilling-injured fruit to a physical disruption of the cytoskeleton due to low temperature exposure. Similarly, ethylene production during the shelf-life that follows coldstorage of persimmon has been also associated with chilling injury alleviation by 1-MCP treatment (Orihuel-Iranzo, Miranda, Zacarías & Lafuente, 2010).

It has been described that the chilling injury of 'Fuyu' is associated with a redox state alteration due to chilling stress, and that the protective effect of 1-

MCP treatment is associated with increased ROS scavenging enzymes activity (Zhang et al., 2010). The CI reduction by CO₂ treatment observed in this work may also be related to changes in the redox system since, in other persimmon cultivars, CO₂ treatment has been reported to alter the redox balance to result in increased ROS scavenging enzymes activity (Novillo et al., 2014). It is known that vegetables have a complex system which can respond to different levels of stress exposure, in which mild exposure generally enhances the cell's capacity to resist further or future exposure to either a given particular stress or another stress type (Toivonen, 2003). Polenta et al. (2005) reported ethanol accumulation to be a good variable for monitoring anoxic treatments since it can reflect stress exposure intensity. In the present study, the highest accumulation of ethanol and acetaldehyde was observed after the 36-hour treatment, which indicates that the longer the treatment, the more intense stress becomes. The fact that the 36-hour treatment led fruit to the highest anoxia stress, and as it was also the treatment that resulted in most marked chilling injury reduction, corroborate that anoxia stress exerts a protective effect when fruits are later exposed to chilling stress. Despite the accumulation of ethanol associated to high CO₂ treatments, 58.32 mg/100 mL in fruit treated for 36h, off-flavours were not detected by the sensory panel. Deterioration of taste in persimmon has been related to accumulation of ethanol to levels exceeding 75 mg/100 mL by Ben-Arie, Zutkhi, Sonogo and Klein (1991). Moreover, Arnal, Besada, Navarro and Salvador (2008) reported that ethanol concentration close to 120 mg/100 mL had not a negative effect on flavour of 'Rojo Brillante' persimmon.

Besides metabolic status, the chemical tissue composition at the time of chilling can affect tissue resistance to low temperatures. Chilling-resistant tissues tend to present a higher degree of unsaturation of fatty acids in membrane lipids than chilling-sensitive tissues (Cao, Yang, Cai & Zheng, 2011). While studying changes in volatile compounds associated with the deastringency treatment of nine persimmon cultivars, Besada, Sanchez, Salvador and Granell (2013) reported that, besides acetaldehyde and ethanol dramatically increasing, the high CO₂ treatment had additional effects and all the studied cultivars showed higher concentrations of lipid-derived aldehydes after treatment if compared to harvest time. This increment in lipid-derived aldehydes suggests an effect of high CO₂ treatments on membrane lipids, which may also contribute to confer chilling tolerance to tissues.

In summary, the short-term high CO₂ treatments in 'Fuyu' persimmon based on fruit exposure to high CO₂ concentrations alleviate flesh gelling and fruit darkening, which are the main CI symptoms in this cultivar, during cold storage and subsequent shelf-life.

The microstructural study allowed us to describe changes in the parenchyma structure associated with flesh gelling as the complete loss of cell structure and cellular material output. Short-term high CO₂ treatments alleviated flesh gelling by preserving the integrity of cell walls and the plasmalemma. The treatment based on high CO₂ exposure for 36h led to the greatest chilling injury alleviation. Therefore this treatment must be considered a useful tool for chilling injury control of 'Fuyu' persimmon.

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CHAPTER VII

Response of ‘Fuyu’ persimmons to disinfesting ethyl formate fumigation and 1- MCP treatment

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Abstract

Ethyl formate (EF) is a natural plant volatile and possible alternative to methyl bromide (MeBr) in the postharvest insect control of persimmon fruit. EF is available as a mixture in liquid carbon dioxide (Vapormate™), and it is effective against wide range of insects in persimmons such as mealybug, thrips, and a range of other hitchhikers pests. This technology allows greater market access opportunities for persimmon fruit. Nevertheless, nowadays the treatment is not optimised since persimmon industries have observed some softening related to chilling injury (CI) after cold storage in fruit treated with EF. This issue leads to restricted market access opportunities to meet export. Therefore, our objective was examined the effect on fruit using a range of EF application conditions (0-2% for 2 and 4h) following application of cold storage period. Furthermore, since 1-methylcyclopropene (1-MCP) has a potential effect on reducing CI in persimmon fruit, the potential effect of 1-MCP applied in combination with EF was also examined. To this end, fruit measurements such as colour and firmness change, respiration rate, ethylene production, volatiles compounds, formic acid residue and CI disorders were analysed. Our results showed that the application of 1-MCP in combination with EF solved the problems associated to EF, regardless of the concentration applied.

Keywords: Ethyl formate, Postharvest desinfestation, Fumigation, Persimmon, Softening, Chilling Injury

1. Introduction

Persimmons are a ready host to a wide range of insects and this limits access to international markets. The presence of mealybug, thrips, and a range of other hitchhiker pests are major constraints to marketing New Zealand persimmons (*Diospyros kaki* L.). New Zealand's export persimmons are frequently subjected to postharvest disinfestation techniques such as hot water immersion (Lay-Yee et al., 1997), cold storage (Jamieson et al., 2009). While methyl bromide (MeBr) can be used when pests are discovered, its use is unlikely to continue, and more importantly, fruit quality is significantly reduced. Thus there is a need for alternative postharvest disinfestation treatments, and fumigation is a relatively simple treatment to apply commercially.

The continuing loss of chemicals such as organophosphates and methyl bromide for pest control has increased commercial interest in other soft technologies that allow greater market access for persimmon fruit. Ethyl formate (EF) has been used as an alternative method as a postharvest disinfestation treatment for persimmons. EF is a Generally Recognized as Safe (GRAS) plant volatile compound that can be applied as a fumigant to disinfect fruit and vegetables and it is effective against a wide range of surface pests, and breaks down into formic acid and ethanol (Jamieson et al., 2009). A GRAS compound is determined by the US Food and Drug Administration (US FDA) and such compounds are considered safe for use with human food (Chhagan et al., 2013). The advantage of treatments utilizing GRAS compounds is that they are already accepted by the United States Congress as a series of strict criteria have been satisfied. EF is flammable and explosive when mixed with air at concentrations to kill pests, but formulations in CO₂ reduce this risk significantly (Lawrence 2005; Chhagan et al., 2013). EF is available and registered in New Zealand as Vapormate™ for use in kiwi fruit, table grapes, strawberries, pineapples, banana, capsicum, onion, lettuce, kumara, rhubarb and stored grains. Vapormate™ is formulated in CO₂ (containing 16.7% by weight ethyl formate) and it has been trialed on mainly surface insects such as spider mite, western flower thrips, omnivorous leafroller, aphids, mealy bugs, black widow spiders on horticultural products (Krishna et al., 2002; Simpson et al., 2004; De Lima 2006; Simpson et al., 2007; van Epenhuijsen et al., 2007; Damcevski et al., 2010; Finkelman et al., 2010).

Following the issues in the persimmon industry, the fruit treated with EF showed some softening related to chilling injury (CI) after cold storage so it

looks like EF treatment emphasizes the CI issues when fruit is ready for marketing. This leads to restricted market access opportunities to meet export (e.g. Asia, Australia, USA) and domestic market requirements for New Zealand persimmons.

1-Methylcyclopropene (1-MCP) is a chemical applied as a gas that strongly inhibits ethylene action. It has been showed in several studies the potential effect of 1-MCP reducing CI in persimmon fruit during storage (Woolf et al., 1997; Salvador et al., 2004; Krammes et al., 2005). However, the potential effect of 1-MCP treatment in combination with EF desinfestation treatment has not been examined.

Although many researchers have tested the efficacy of EF as a fumigant for pest control, few reports have described the effect on fruit quality. Besides, it is noteworthy that no studies have been performed on quality and tolerance of persimmon fruit. Taking into account the above considerations this study examined the effect on fruit using a range of EF application conditions (time and doses of EF) following application of cold storage period. In addition this research aimed to determine the potential effect of 1-MCP applied in combination to reduce softening and CI disorders by EF.

2. Materials and methods

2.1. Plant material and experimental design

Persimmon (*Diospyros kaki* L.) cv. Fuyu fruit were harvested on 26 May 2014 from commercial orchards in East Coast of New Zealand's North Island, Gisborne. Fruit were commercially packed to export standards. Fruit were held in the packhouse at ambient temperatures overnight prior grading and packing to export standards. Fruit were distributed into 350 trays of 20-count size. On the same d of packing, fruit were transported to the Mt Albert Research Centre (Auckland), and fruit quality measurements were assessed to evaluate fruit at harvest time. Later, 175 trays were subjected to 1-methylcyclopropene treatment (1-MCP). The following d all the fruit were subjected to ethyl formate (EF) fumigation. Fruit treated with EF were compared to untreated control fruit that remained at 15 °C through the treatments.

Treated and untreated fruit were sealed in modified atmosphere (MA) bags and cool-stored (≈ 0 °C) for 40 d. Trays were placed on pallets in piles of seven high, with ≈ 3 cm gaps between stacks to maximize airflow. The air in cool room was set at -0.5 °C, aiming for a fruit temperatures of < 0 °C.

After cold stored period, the MA bags were removed and fruit were transferred to 20°C for 5 d simulating a shelf-life period. Fruit quality measurements were assessed during d 1, 3 and 5 of shelf-life (20 °C) on selected treatments. Chilling injury (CI) disorder was evaluated after 5 d at 20 °C over all treatments. Furthermore the percentage of CO₂ and O₂ were measured before removing MA bags to transfer the fruit to 20 °C. The average of % O₂/% CO₂ inside the bags was 1.9/6.8 and showed not pattern of effect of EF or 1-MCP treatment.

2.2. Treatments

2.2.1. 1-Methylcyclopropene (1-MCP) treatment

It was completed using a 4 m³ polyethylene tent within a commercial store room set to operate at 20°C. Two battery-operated Coleman® fans (with approx. 15 cm blades) were placed inside each tent for air circulation. The required dose (625 ppb) was achieved by weighing 148 mg of SmartFresh powder (active ingredient 3.8%) into 250 mL Schott bottles, and activated following the addition of ~ 100 mL of tepid water and a quick, gentle shake. Once the SmartFresh treatment was activated, the tent was sealed using duct tape and left for 24 h before opening.

2.2.2. Ethyl formate (EF) treatment

EF treatments were applied at calculated target doses of 0, 0.5, 1, 1.5 and 2% EF for 2 and 4 h. The treatments were carried out in the Volatile Treatment Facility (VTF) at Plant & Food Research (PFR) Auckland. Twenty eight identical 76.8 L steel, gas tight chambers were used for this trial in a controlled temperature room. Fruit were treated within metal baskets held within each of the chambers. The amount of EF and CO₂ delivered to each chamber was controlled by the user input into the computer program. A CO₂ gas stream (10 L min⁻¹) was passed through a heated bead bath (75°C) and pure liquid EF (Merck, 98%) was delivered using a micro-dispenser (INKX0523050A, The Lee Company, Westbrook CR, USA) into the heated gas stream. The gas was

again passed through the heated bead bath to volatilise the EF before delivery into the chamber. The chambers filled automatically one after the other and were purged of EF once the treatment time was completed. EF was monitored in each chamber with 50 μL samples from the chambers and injected into a gas chromatograph (GC) unit (Philips® PYE UNICAM PU4500 Chromatograph). The calculated target and actual concentrations of EF applied during the treatments are presented in table 1. Carbon dioxide was also monitored by extracting a 1mL sample and injecting into a carbon dioxide analyser with Hewlett Packard® integrator. The treatment of 0% of EF was applied in order to evaluate the effect of CO_2 due the treatment is commercially applied in combination with carbon dioxide (Mimicking VAPORMATE™ formulation).

Treatments	Calculated EF	Calculated EF	Actual EF (%)	
	(g/m3)	(%)	Initial ¹	Final ²
Control	Untreated	-	-	-
1-MCP	Untreated	-	-	-
0 % EF 2h	0,00	0,00	0,00	0,00
0 % EF 4h	0,00	0,00	0,00	0,00
0,5 % EF 2h	97,56	0,50	0,48	0,32
0,5 % EF 4h	97,56	0,50	0,49	0,23
1 % EF 2h	195,12	1,00	0,95	0,61
1 % EF 4h	195,12	1,00	0,90	0,44
1,5 % EF 2h	292,68	1,50	1,44	0,84
1,5 % EF 4h	292,68	1,50	1,34	0,60
2 % EF 2h	390,24	2,00	1,88	1,22
2 % EF 4h	390,24	2,00	1,76	0,76
0 % EF-MCP 2h	0,00	0,00	0,00	0,00
0 % EF-MCP 4h	0,00	0,00	0,00	0,00
0,5 % EF-MCP 2h	97,56	0,50	0,48	0,35
0,5 % EF-MCP 4h	97,56	0,50	0,49	0,22
1 % EF-MCP 2h	195,12	1,00	0,94	0,82
1 % EF-MCP 4h	195,12	1,00	0,88	0,44
1,5 % EF-MCP 2h	292,68	1,50	1,47	1,20
1,5 % EF-MCP 4h	292,68	1,50	1,35	0,65
2 % EF-MCP 2h	390,24	2,00	1,84	1,47
2 % EF-MCP 4h	390,24	2,00	1,78	0,86

Table 1. Target and actual concentrations of ethyl formate (EF) and CO_2 applied during the fumigation through treatments

¹The initial concentration of EF measured 10-20 minutes after the start of the treatment.

²The final concentration of EF measured at the conclusion of the treatment.

2.3. Fruit measurements

Fruit assessments during shelf-life period (d 1, 3 and 5) were analyzed on untreated fruit (control and 1-MCP fruit) and selected EF treatments (1 and 2% for 2 and 4 h) treated/untreated with 1-MCP. External skin colour, firmness and CI disorders were analysed over all treatments on the d 5 of shelf-life.

2.3.1. External skin colour

Skin colour was measured on 20 fruit per treatment, using a Minolta (Model CR-400 Ramsey, NY, USA) on three sides of the fruit. Hunter parameters 'L', 'a', 'b', were measured and the results were expressed as colour index: $CI=1000a/Lb$ (Salvador et al., 2004).

2.3.2. Fruit firmness

Firmness was determined using a Fruit Texture Analyser (Guss, model GS14, South Africa) fitted with a 7.9 mm Effegi™ penetrometer probe. The probe was driven into the flesh at 5 mm/s to a depth of 7.9 mm, and the maximum force recorded as the firmness value. Two measurements were made per fruit on pared surfaces on opposite sides of the fruit over 20 fruit per treatment. The results were expressed as Newton (N).

2.3.3. Respiration rate and ethylene production

It was determined on an individual fruit basis (6 fruit per treatment) using a flow through system. Carbon dioxide was measured using an infra-red CO₂ transducer (Servomex Autotech Engineering, United Kingdom) with nitrogen as the carrier gas and ethylene was measured using analysing the sample by flame ionisation gas chromatography (Hewlett Packard 5890 series II, fitted with a glass activated alumina column). Respiration rate was expressed as $\text{mg kg}^{-1} \text{h}^{-1}$ and ethylene production as $\mu\text{L kg}^{-1} \text{h}^{-1}$.

2.3.4. Volatile analysis

Acetaldehyde and ethanol production were determined on an individual fruit (9 fruit per treatment) by taking two cylindrical plugs from each side of the fruit, placing the plugs into a 60 mL syringe and applying a vacuum for one minute, after 1 min the vacuum was released and a 1 mL headspace sample

withdrawn. Analysis of 1 mL sample was done by the injection into a gas chromatograph (Pye unicam Model PU4500 fitted with a Carbowax column and flame ionisation detector). The results were expressed as nmol/g.

2.3.5. Formic acid analysis

A formic acid detection kit (Megaenzyme International Ireland, K-FORM, Wicklow, Ireland) was used to analyze the fruit at harvest time and at the end of shelf-life period (d 5). Samples from 9 fruit per treatment were cut into small pieces and frozen with liquid nitrogen to be ground and kept at -80 °C. Three grams of frozen fruit powder was blended with 4 mL of distilled water, stirred for 15 min and the homogenates were centrifuged at 10000 rpm for 20 min at 4 °C. Supernatants were filtered and combine with NAD⁺ and formate dehydrogenase solution in a buffer at pH 7.6. In the presence of NAD⁺, formic acid is oxidized to CO₂ and NADH by formate dehydrogenase. The concentration of NADH formed was measured with a spectrophotometer (Biochrom Libra Instruments, S22 UV, Holliston, MA) at 340 nm. Formic acid content was determined using a standard curve and was expressed as µg g⁻¹.

2.3.6. Chilling injury (CI) disorder

CI was evaluated after 5 d at 20 °C over all the treatments and it was assessed subjectively using methods develop over time but originally based on MacRae (1987). Fruit categorised according to the fruit firmness and amount of “gelling” detected within the flesh when cut through the equator.

0 = Very firm fruit with little give, no gelling, flesh feels coarse when rubbed with finger

1 = Flesh with slight give, no gelling, feels slightly slippery to the touch

2 = Flesh with moderate give, slight gelling (darkening of flesh), feels slippery to the touch

3 = Flesh generally soft, gelling (darker orange) of ≈ 50% of the surface, flesh slippery to the touch

4 = Flesh soft, gelling (darker orange) of ≈ 75% of the surface, flesh slippery to the touch

5 = Flesh soft, gelling (darker orange) of \approx 100% of the surface, flesh slippery to the touch

A rating of more than 2 was considered commercially unacceptable; however, a rating of 2 is only marginally acceptable.

Most disorders are presented in terms of the proportion of unacceptable fruit (i.e. \geq 2), which is referred to as “severity”. Alongside CI evaluation, skin colour and firmness were also assessed in all the treatments.

2.4. Statistical analysis

Data were statistically analysed with the Statgraphics Plus version 5.1; Manugistics Inc., Rockville, MD, USA), using multifactor analysis of variance (ANOVA). Statistical significance was judged at the level ≤ 0.05 . The Fisher’s Protected Least Significant Difference (LSD) test was used to separate means. The chilling injury disorder was calculated for each tray and analysed based on an arcsine transformation: arcsine (sqrt (incidence)). Shown values are non-transformed means.

3. Results and discussion

Last season, the New Zealand persimmon industry used VAPORMATTM formulation, at dose of 240 g m⁻³ (which is approximates to 1.24%) for 4 h at 15 °C and the fumigation treatment was effective in killing mealy bugs, two spotted and tydeid mites, red back spider, light brown apple moth and western flower thrips. Nevertheless the fumigation treatment showed some softening for fruit after long cold storage. In order to determine the effect of EF on the fruit quality and the potential effect of 1-MCP applied in combination; several treatments with a range of concentrations and durations have been evaluated.

Fig.1 shows the firmness (Fig. 1A) and colour index (Fig. 1B) values after 5 d at 20 °C following 40 d of cold storage of persimmon treated and untreated with 1-MCP subjected to fumigation of different concentrations of EF. As previously seen by the persimmon industries, the application of EF induced fruit softening. The fruit treated only with EF showed a decreased on firmness

comparing to untreated fruit. It is noteworthy that there was no significant difference on firmness values between different concentrations and duration of the treatment. The fumigation of EF would be carried to soften fruit regardless to the concentration used. Nevertheless, all treatments treated with 1-MCP in combination with EF showed similar firmness values (over 60 N) to fruit that was only treated with 1-MCP; indicating that 1-MCP was effective on maintain firmness on fruit treated with EF, independently of concentration and duration.

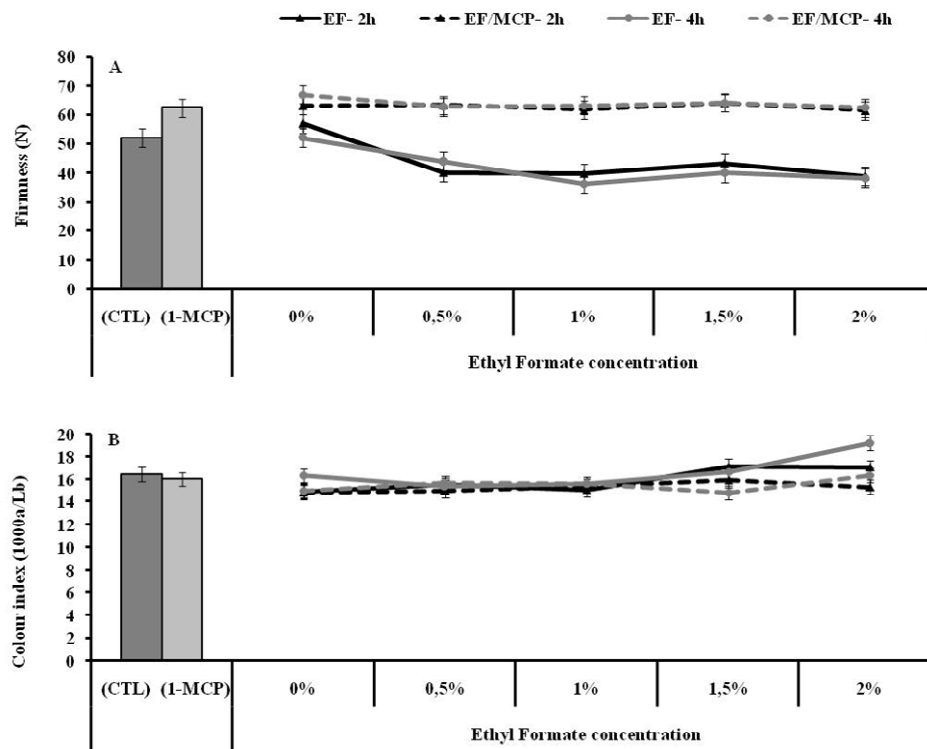


Fig. 1. Fruit firmness (A) and external colour index (B) values in persimmon fumigated with doses of 0-2% ethyl formate (EF) for 2 and 4 h and untreated fruit (CTL and 1-MCP) then stored for 40 d at 0°C in under MA, and 5 d shelf-life at 20 °C (removed from MA bags). Vertical bars indicate LSD intervals ($P<0.05$).

Studying the firmness evolution during shelf-life was possible to observe that fruit treated with 1-MCP showed slight changes in firmness values. The fruit only treated with EF did not show differences among treatments at day 1; however at day 3 the firmness started to decrease comparing to the other treatments with 1-MCP or the untreated fruit (data not shown).

Taking into account the external skin colour of the fruit, no significant difference was observed between fruit treated combining 1-MCP plus EF and fruit treated with only EF. The EF treatment did not show effect on skin colour in the most of treatments (Fig. 1B); however the treatment subjected to 2% of EF for 4h showed the highest colour index (19.2), showing statistical differences comparing to others at day 5 at 20 °C. Considering the evolution of colour during shelf-life, there was an increase of colour index from day 1 to day 5 with no significant difference among treatments (data not shown). Unlike persimmon fruit, studies with EF and its impact on strawberry and citrus fruit quality showed that concentrations up to 2.4% of EF did not show an impact on the skin colour and fruit firmness; even after shipping and cold storage of 5 weeks (Simpson et al., 2004; Pupin et al., 2013).

Fig. 2 shows the CI disorders, represented as percentage of incidence (Fig. 2A) and unacceptable fruit (Fig. 2B). The 1-MCP treated fruit and treatments combining 1-MCP and EF showed similar values of percentage of CI incidence with no statistical difference among treatments. However, control fruit and treatments with EF showed higher percentage of CI incidence than treatments with 1-MCP. It is noteworthy that no differences were found between untreated fruit (control) and 0% of EF (only application of CO₂ with no EF), indicating that the way in which EF is applied as VAPORMATTM did not have negative effect on CI incidence. Simpson et al. 2004 showed no effect on strawberry damages after the inclusion of CO₂ with EF fumigation. Furthermore, studies carried out by Chhagan et al. 2013 in apricot fruit resulted in comparable fruit quality results using CO₂ or N₂ as carrier gas. All fruit treated with EF showed higher percentage of CI incidence than the untreated fruit and treatments combining EF with 1-MCP. The EF treatments from 0.5 to 2% showed similar CI incidence values, over 50%; with no significant differences between concentration of EF and duration of application.

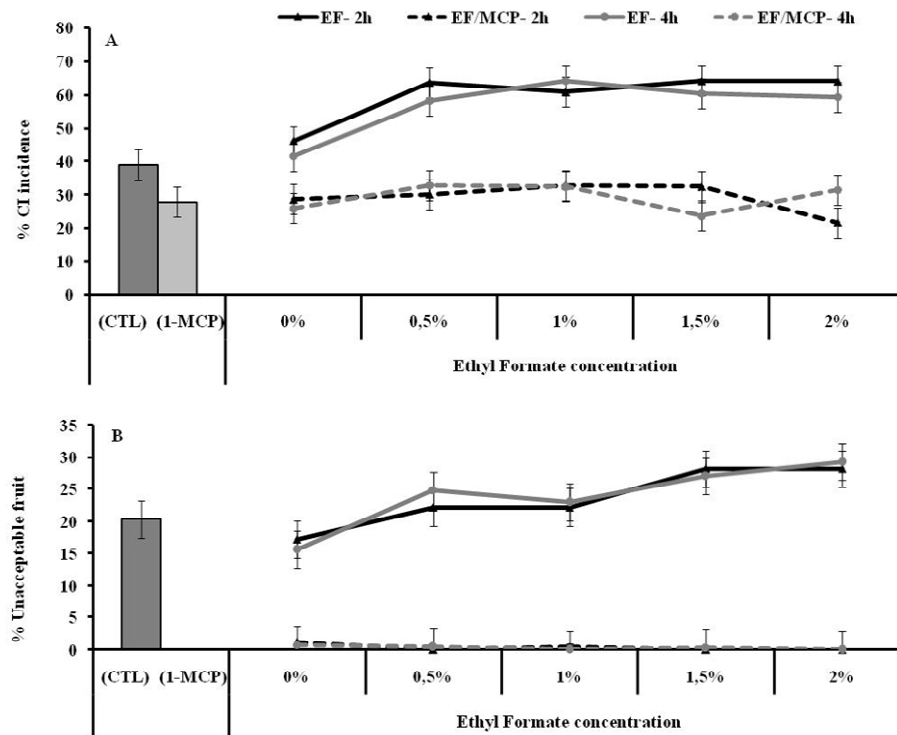


Fig. 2. Chilling injury (CI) disorder as percentage of CI incidence (A; > 0 rating) and percentage of unacceptable fruit (B; ≥ 2 rating) in persimmon fumigated with doses of 0-2% ethyl formate (EF) for 2 and 4 h and untreated fruit (CTL and 1-MCP) then stored for 40 d at 0°C in under MA, and 5 d shelf-life at 20 °C (removed from MA bags). Vertical bars indicate LSD intervals ($P < 0.05$).

The proportion of unacceptable fruit is referred to as severity, becoming in unmarketable fruit. The fruit treated only with 1-MCP and the treatments combining EF and 1-MCP showed no unacceptable fruit or values close to 0% (Fig. 2B); highlighting that 1-MCP led to maintain acceptable fruit independently to EF concentration or duration applied. It is important to highlight that slight changes were observed among EF treatments without 1-MCP application; only treatments with 1.5 and 2% of EF showed a higher percentage of unacceptable fruit than untreated fruit, with values over 27% of unacceptable fruit. The high CI incidence may increase the percentage of

unacceptable fruit. Thus fruit treated with EF without 1-MCP application showed a higher CI incidence that may lead to an increase in unacceptable fruit whether cold storage and shelf-life are extended.

Although, studies on citrus and apricot fruit did not show fruit damage after EF fumigation (Pupin et al., 2013; Chhagan et al., 2013); other studies conducted in strawberries and table grapes have reported damage to fruit following EF treatments; resulting in calyx damage and rachis browning (Simpson et al., 2004; Simpson et al., 2007; DeLima et al., 2006). In the case of persimmon fruit, the results from the present research are indicating an extensive damage to the fruit that increase the CI incidence and resulting in a drastic softening and unmarketable fruit.

After measuring the respiration rate (Fig. 3A) and ethylene production (Fig. 3B) during shelf-life in selected treatments, it was possible to observe a slight decreased in CO₂ production from d 1 to d 5 at 20°C with no statistical difference during shelf-life (Fig. 3A). Moreover, there was no significant difference between treatments of 1% of EF for 2 and 4 h and the same treatments combining 1-MCP. Nevertheless, treatments combining 2% EF for 2 and 4 h with 1-MCP reduced the respiration rate comparing to the same treatments with no application of 1-MCP; indicating an effect of 1-MCP on reducing CO₂ production when it was applied in combination with high concentrations of EF. The ethylene production during shelf-life remained unchanged for the most of treatments (Fig. 3B); excluding treatment of 2% of EF for 2 and 4 h which showed values over 0.04 μL kg⁻¹ h⁻¹. As seen in respiration rate, the application of 1-MCP combined with high concentration of EF reduced the ethylene production. Thus the uses of high concentrations of EF are able to increased CO₂ and ethylene production but this rise could be offset by 1-MCP application.

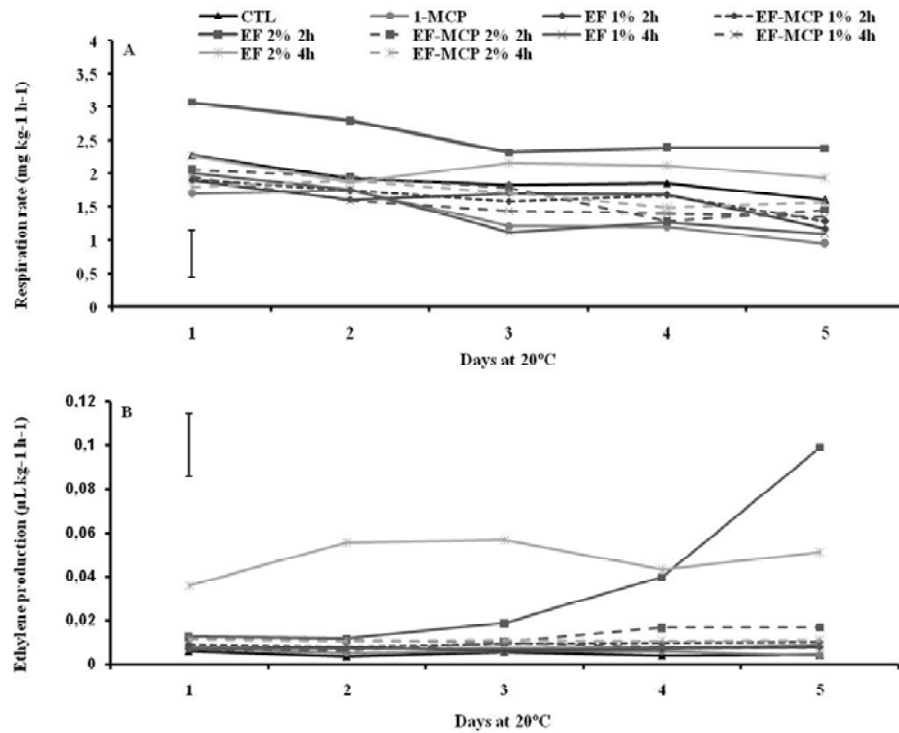


Fig. 3. Respiration rate (CO₂ production) (A) and ethylene production (B) in persimmon fumigated with doses of 1 and 2% ethyl formate (EF) for 2 and 4 h and untreated fruit (CTL and 1-MCP) then stored for 40 d at 0°C in under MA, and 5 d shelf-life at 20°C (removed from MA bags). Vertical bars indicate LSD intervals ($P<0.05$).

Regarding acetaldehyde and ethanol production during shelf-life; the production of both volatile compounds remained unchanged from d 1 to d 5 at 20°C, with no significant difference among treatments; excluding treatments of 2% of EF for 2 and 4 h that showed higher values of acetaldehyde production (over 2.8 nmol g⁻¹) than the other treatments at d 5 (data not shown). It has been reported in several studies in strawberry and citrus fruit of EF fumigation that the levels of volatile compounds remained unchanged using concentrations lower than 0.8% of EF, but the volatiles were greater than untreated fruit with increased exposure to EF (Pupin et al., 2013; Simpson et al., 2004). The increase of volatiles and residues from fumigation treatment may lead to bad

taste and organoleptic quality loss in the fruit. Considering that EF breaks down to ethanol and formic acid and trace residues have been found on treated products such as, wheat, barley, oats and date or apple fruit (Desmarchelier and Ren, 2009; Learmonth, 2012; Bessi et al., 2013); formic acid residues have been evaluated at the end of shelf-life (d 5). The values of formic acid found ranged from 2 to 2.68 $\mu\text{g g}^{-1}$; showing no significant difference between fruit treated with EF and fruit at harvest time or control fruit (data not shown); corroborating that fruit treated with EF did not show bad flavours after an informal sensory evaluation. Thus, EF fumigation applied prior to export fruit did not show residues during shelf-life period with no negative consequences on the sensory attributes of the fruit. These findings are in accordance to those of Pupin et al. 2013 who reported that no formic acid residues remained after EF fumigation, showing no negative effect on citrus fruit flavour.

4. Conclusions

To summarize, the present paper reports that EF fumigation which is employed to disinfest fruit and vegetables as quarantine pest to export New Zealand's persimmons, has a negative effect on the fruit quality resulting in a fruit firmness loss and emphasizing the CI disorders. The application of 1-MCP in combination with EF solved the problems associated to EF, regardless of the concentration applied. The results obtained and the knowledge acquired can be useful for the application of EF, as Vapormate™, as a fumigant for quarantine pest to export New Zealand's persimmons with no negative effects on the fruit quality. This fact leads to market access opportunities to meet export and domestic market requirements for New Zealand persimmons.

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CHAPTER VIII

Is there a relationship between ethyl formate and ethylene biosynthesis in persimmon fruit?

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Reference: *Pending submission*

Abstract

Ethyl formate (EF) is a ‘generally recognized as safe’ (GRAS) compound that can be applied as fumigant to disinfest fresh produce. It can be considered as a possible alternative to methyl bromide (MeBr) in the postharvest insect control of persimmon fruit allowing wider market access. Previous studies conducted in persimmon fruit have showed a significant loss of quality due its use, resulting in fruit softening during postharvest distribution. Taking into account the assumption that fruit softening is mediated by ethylene; our aim was to determine the involvement of ethylene on the fruit softening induced by ethyl formate. To this end the relative expression of the ethylene biosynthesis and cell wall genes using RT-qPCR analysis were investigated in fruit treated/untreated with ethyl formate (2% for 4h); also fruit treated with 1-methylcyclopropene (1-MCP) was investigated since it is considered a strong inhibitor of ethylene action. Furthermore, fruit quality assessments such as external skin colour, flesh firmness, respiration rate, ethylene, ethanol and acetaldehyde production and residual formic acid were analyzed during shelf-life (14 d at 20°C). Our results showed that ethyl formate treatment increased the relative expression of *DkACO1*, *DkACO2*, *DkACS2*, *DkERS1* and *DkETR2* genes and fruit showed an increased ethylene production leading to significant fruit softening.

Keywords: Ethyl formate, Ethylene biosynthesis genes, Persimmon, Softening

1. Introduction

The postharvest methods commonly used for pest control in horticulture products have been treatments based in organophosphates and methyl bromide. However, there are a number of problems associated with the use of organophosphates and methylbromide fumigation, such as its toxicity to humans or food sources. In addition there are concerns about the future availability and cost of these fumigants; even more after the phase out of methylbromide for all uses except quarantine treatments. So, the continuing loss of chemicals such as organophosphates and methyl bromide for pest control has increased the commercial interest of the horticultural industries in other soft technologies that allow greater market. Thus, in the recent years, it has been evaluating the ethyl formate as alternative method for postharvest disinfestation. Ethyl formate is a GRAS (Generally Recognized as Safe) plant volatile compound that can be applied as a fumigant to disinfect fruit and vegetables. It is effective against a wide range of surface pests, and breaks down into formic acid and ethanol (Jamieson et al., 2009). The advantage of treatments utilizing GRAS compounds is that they are already accepted by the United States Congress as satisfying a series of strict criteria. Ethyl formate is available and registered in New Zealand, Australia, Korea, Philippines, Malaysia and Israel as Vapormate™ for its use on horticulture products. Vapormate™ is formulated using CO₂ (containing 16.7% by weight ethyl formate) and it has been trialed on mainly surface insects such as spider mite, western flower thrips, omnivorous leafroller, aphids, mealy bugs, and black widow spiders on horticultural products (Krishna et al., 2002; Simpson et al., 2004; De Lima 2006; Simpson et al., 2007; van Epenhuijsen et al., 2007; Damcevski et al., 2010; Finkelman et al., 2010; Cho et al., 2012; Chhagan et al., 2013; Griffin et al., 2013).

In the case of persimmon fruit, in the last years the improved knowledge of its storage and management leads to great opportunities to increase their export industry. Thus, the use of ethyl formate as a disinfestation treatment may provide significant opportunities for the persimmon industries to meet export and domestic market requirements. Furthermore, in persimmon fruit, the fumigation with ethyl formate has been effective for the control of two-spotted spider mites, crape-myrtle scale, mealy bugs, western flower thrips and persimmon fruit moth (Cho et al., 2012)

However, in our previous studies using ethyl formate for fumigation of persimmon fruit there was significant fruit softening during postharvest

distribution (unpublished data). This softening in persimmon fruit represents a significant loss of quality and consequently important economic losses. It is well known that fruit softening is one of the ripening processes that is most sensitive to ethylene. In fact, several studies have demonstrated the relationship between ethylene production and fruit softening (Itamura et al., 1997; Lelièvre et al., 1997). Moreover, molecular studies have demonstrated the involvement of 1-aminocyclopropane-1-carboxylic acid synthase (*DkACS*) and oxidase (*DkACO*) (Zheng et al., 2005) and the ethylene receptor genes *DkERS1* and *DkETR2* in persimmon fruit ripening (Pang et al., 2007). Furthermore, methods to extend shelf-life are based on the control of ethylene action, such as 1-methylcyclopropene (1-MCP). 1-MCP is a strong inhibitor of ethylene action, and its effect in the control of persimmon ripening has been widely evaluated (Salvador et al., 2004; Luo, 2007).

In view of the foregoing, the present research aimed to study the involvement of ethylene on the fruit softening induced by ethyl formate; to this end the expression of the ethylene biosynthesis and ethylene receptor genes *DkACO1*, *DkACO2*, *DkACS2*, *DkETR1*, *DkETR2* and *DkERS1* using qPCR analysis have been evaluated. In addition, it has been addressed fruit quality assessments such as external skin colour, flesh firmness, respiration rate, ethylene production, volatile production and residual formic acid. Furthermore in order to understand aspects of softening the expression of the cell wall metabolism genes *DkEXP3*, *DkPG1*, *DkXTH1* and *DkXTH2* have been explored. In this research, fruit treated with ethyl formate have been evaluated during two weeks at 20°C after fumigation, and compared to untreated fruit (control) and fruit treated with 1-MCP.

2. Materials and methods

2.1. Plant material

Persimmon (*Diospyros kaki L.*) cv. Fuyu fruit were harvested on 26 May 2014 from commercial orchards at Gisborne on the east coast of New Zealand's North Island. Fruit were held in the packhouse at ambient temperatures overnight prior to grading and commercial packing to export standards.

Thirty six trays of 20-count size fruit were transported on the same day to the Mt Albert Research Centre (Auckland), where fruit quality measurements were done to evaluate fruit maturity characteristics at harvest. Twelve trays of fruit were subjected to ethyl formate (EF) fumigation and another 12 trays were subjected to 1-methylcyclopropene (1-MCP) treatment. Untreated persimmon fruit (control) remained at 20°C throughout the treatments.

2.2. Treatments

2.2.1. Methylcyclopropene (1-MCP) treatment.

It was applied using a 4m³ polyethylene tent within a commercial store room set to operate at 20°C. Two battery-operated Coleman® fans (with approx. 15 cm blades) were placed inside each tent for air circulation. The required dose (625 nL L⁻¹) was applied by weighing 148 mg of SmartFresh powder (active ingredient 3.8%) into 250mL Schott bottles, and activating it by the addition of ~100 mL of tepid water with, gentle shaking. Once the SmartFresh treatment was activated, the tent was sealed using duct tape and left for 24 hours before opening and fruit removal.

2.2.2. Ethyl formate treatment

The treatment was applied at calculated target dose of 2 % ethyl formate in CO₂ for 4 hours in the Volatile Treatment Facility (VTF) at Plant & Food Research (PFR) Auckland. Nine identical 76.8 L steel, gas tight chambers were used for this trial in a controlled temperature room. Fruit were treated within metal baskets held within each of the chambers. The amount of ethyl formate and CO₂ delivered to each chamber was controlled by a computer programme. A CO₂ gas stream (10 Lmin⁻¹) was passed through a heated bead bath (75°C) and pure liquid ethyl formate (Merck, 98%) was delivered using a micro-dispenser (INKX0523050A, The Lee Company, Westbrook CR, USA) into the heated gas stream. The gas was again passed through the heated bead bath to volatilize the ethyl formate-CO₂ mixture before delivery into the chamber. The chambers filled automatically one after the other and were purged of ethyl formate once the treatment time was completed. Ethyl formate concentration was monitored in each chamber using 50 µl samples of gas from the tent injected into a gas chromatograph (GC) unit (Philips® PYE UNICAM PU4500 Chromatograph). The CO₂ was also monitored by extracting a 1mL sample and

injecting into a carbon dioxide analyser (Philips® PYE UNICAM PU4500 Chromatograph) using a Hewlett Packard® H03395 integrator.

2.3. Fruit quality assessments

Twenty fruit per treatment were sampled at commercial harvest to assess maturity characteristics and again immediately after ethyl formate treatment (0 day) 1, 4, 7, 10 and 14 days after treatment. Measurements taken at each assessment were fruit firmness, external skin colour, respiration rate, ethylene production, and volatile analysis for acetaldehyde and ethanol. A tissue sample was frozen in liquid nitrogen and stored at -80 °C for formic acid analysis and RNA extraction for gene expression analysis.

External skin colour was measured using a Minolta CR-400 (Ramsey, NY, USA) on three sides of the fruit. Hunter parameters 'L', 'a', 'b', were measured and the results were expressed as colour index: $CI=1000a/Lb$ (Salvador et al., 2004; Salvador et al., 2007).

Fruit firmness was determined using a Guss fruit texture analyser (model GS14, South Africa) fitted with a 7.9 mm Effegi™ penetrometer probe. The probe was driven into the flesh at 5 mm s⁻¹ to a depth of 7.9 mm, and the maximum force recorded as the firmness value. Two measurements were made per fruit on paped surfaces on opposite sides of the fruit with the results expressed as Newton (N).

Respiration rate and ethylene production were determined by withdrawing a 1 cm³ sample of headspace gas after sealing each fruit in a 1000 mL container for one hour. CO₂ was measured using an infra-red CO₂ transducer (Servomex Autotech Engineering, United Kingdom) with nitrogen as the carrier gas. Ethylene was measured by flame ionisation gas chromatography, using a glass activated alumina column (Hewlett Packard 5890 series II). Respiration rate was expressed as μmol kg⁻¹ s⁻¹ and ethylene production as nmol kg⁻¹ s⁻¹. A subset of untreated fruit and fruit treated with ethyl formate were analysed for ethylene production using a flow through system attached to an ETD-300 real time sub-ppb ethylene (C₂H₄) analyzer with a 1665 Hz frequency laser (Sensor Sense B.V. Netherlands). Results were expressed as pmol kg⁻¹ s⁻¹.

Volatile analysis (acetaldehyde and ethanol) was determined by taking two cylindrical plugs from each side of the fruit, placing the plugs into a 60 mL

syringe and applying a vacuum for one minute. After 1 min, the vacuum was released and a 1 mL headspace sample withdrawn. Analysis of this 1 mL sample was done by gas chromatography (PyeUnicam Model PU4500 fitted with a Carbowax column and flame ionisation detector). The results were expressed as nmol g^{-1} .

Formic acid was measured on tissue sampled after one day of ethyl formate fumigation using a kit (Megaenzyme International Ireland, K-FORM, Wicklow, Ireland) according to the manufacturers instructions. Samples from 9 fruit per treatment were cut into small pieces, frozen in liquid nitrogen and stored at -80°C . Three grams of ground tissue were blended with 4 mL of distilled water, stirred for 15 min and the homogenate centrifuged at 10000 rpm for 20 min at 4°C . The supernatant was filtered and mixed with NAD^{+} and formate dehydrogenase solution in a buffer at pH 7.6. In the presence of NAD^{+} , formic acid is oxidized to CO_2 and NADH by formate dehydrogenase. The concentration of NADH formed was measured with a spectrophotometer (Biochrom Libra Instruments, S22 UV, Holliston, MA) at 340 nm. Formic acid concentration was determined using a standard curve and expressed as $\mu\text{g g}^{-1}$.

2.4. RNA extraction and gene expression

Cortical tissue samples from all treatments were collected at harvest and 1, 4 and 7 days following ethyl formate fumigation. At each time point three composite replicate samples of three fruit per replicate were taken frozen in liquid nitrogen and stored at -80°C . For RNA extraction the sample was pooled from three biological replicates composed of three fruits per replicate, RNA extraction was done using a modified version of the method of Chang et al (1993). An aliquot of RNA ($1.5\mu\text{g}$) was reverse transcribed to cDNA using a Tetra cDNA kit (Bioline). Gene expression was analysed using six technical repetitions per sample each comprising a $10\text{-}\mu\text{L}$ reaction of $5\mu\text{L}$ of Mastermix (Roche), $2\mu\text{L}$ of $5\mu\text{M}$ primers (forward and reverse), with $3\mu\text{L}$ of cDNA. Quantitative PCR (qPCR) was done using ACTIN as a reference gene (Akagi et al., 2009). qPCR was completed using a LightCycler 480 (Roche) and analysed using the LightCycler 480 software (Roche).

Primers (Table 1) were designed or modified based on recently published data:

Gene	Forward	Reverse
<i>DkXTH1</i>	GATGAGGCTCTTCTCCAGCA	AGCATGATCGGCTCTGAAGT
<i>DkXTH2</i>	TTCCATATGCGAACAAGCAG	GAAGCAGTGAAGGGAGCTTG
<i>DkACS2</i>	CAATCTGCACCACTGAAGGA	CGCCCTTCCACCTTCTACTT
<i>DkEXP3</i>	CCGTCTCTTTCCAAAGGGTG	TTCTGGACCCCTTGACTGAC
<i>DkPG1</i>	CTTGTAAGAGCAGGGCTCATC	TCTGCAACACAATCCCTTCA
<i>DkACO1</i>	CTCATCAACGATGCCTGTGA	TCCTTGAACCTCTGCTCCAT
<i>DkACO2</i>	TTAATGGCGAAGGGAGAGAA	TTTGTGAGCCTCTCCACTGA
<i>DkETR1</i>	GGCTTTCATTGCGAAGCTAT	GGGGTTTATCCCTGATCCTG
<i>DkETR2</i>	TGCAATTGAGGAATGCTTGA	GGCGAGAGATGAAAACGAAG
<i>DkERS1</i>	TGTGGCTTCTACTGCAAAACC	GGGCTAATACCACAGCCAGA
<i>DkACTIN</i>	CCATCCAGGCTGTTCTGTCT	AGCATGAGGAAGGGCATAAC

Table 1. Sequences of primers used for gene expression analysis. The primers pairs were designed from published persimmon sequence: xyloglucan endotransglycosylase/hydrolase genes (*DkXTH1* and *DkXTH2*) (Nakatsuka et al., 2011); *DkACS2*, *DkACO1*, *DkACO2* (Nakano et al., 2001); ethylene receptor genes (*DkETR1*, *DkETR2*, *DkERS1*) (Pang et al., 2007); expansin gene (*DkEXP3*) (Zhang et al., 2012); actin gene (*DkACTIN*) (Akagi et al., 2009). polygalacturonase (*DkPG1*) (Liu et al. 2009)

2.5. Statistical analysis

Data were statistically analysed with the Statgraphics Plus version 5.1; Manugistics Inc., Rockville, MD, USA), using multifactor analysis of variance (ANOVA). Statistical significance was judged at the level $P < 0.05$. The Fisher's Protected Least Significant Difference (LSD) test was used to separate means.

3. Results and discussion

3.1. Effect of Ethyl Formate on fruit quality assessments

Fig. 1 shows the changes in external skin colour (A) and flesh firmness (B). During the first week of storage slight changes were observed in skin colour and firmness. From day 7, the colour started to increase significantly in fruit treated with ethyl formate. At the same time the firmness decreased in ethyl formate treated fruit; values decreased from 60N to values less than 30N. However, fruit treated with 1-MCP and untreated fruit showed values over 55 N during the 14 days of storage. As seen in previous studies of persimmon fruit, the ethyl formate fumigation treatment induced fruit softening, leading to a significant loss of quality (unpublished data). Unlike persimmon fruit, other studies conducted in strawberries (Simpson et al., 2004), citrus (Pupin et al., 2013) or table grapes (DeLima, 2006) showed no negative impact on fruit firmness after application of ethyl formate concentrations higher than 2%. However, calyx damage was observed in strawberry fruit exposed to ethyl formate concentrations higher than 0.8% (Simpson et al., 2004).

Since ethyl formate breaks down to formic acid and ethanol, formic acid residues were evaluated one day after ethyl formate application. The results showed no accumulation of formic acid after fumigation. The untreated fruit and fruit treated with ethyl formate showed similar levels of formic acid; ranging from 3.88 to 4.63 $\mu\text{g g}^{-1}$ (data not shown), indicating that formic acid had no negative effect on the fruit quality. Nevertheless, the study of the volatile compounds acetaldehyde and ethanol during shelf-life showed an increase of these compounds from day 0 to day 4 in fruit treated with ethyl formate, with values over 0.5 nmol g^{-1} . On day 7, these values decreased to similar levels to those observed in control and 1-MCP treated fruit. The levels of volatile compounds in untreated fruit were unchanged during 14 days of storage, showing values below 0.1 nmol g^{-1} (Fig. 2A and 2B). Pupin et al. 2013 reported that no ethanol and formic acid residue remained in citrus fruit after ethyl formate fumigation for 1 hour at 38g m^3 ; however increased levels of acetaldehyde, ethanol and ethyl acetate were detected in strawberries exposed to ethyl formate (Simpson et al., 2004).

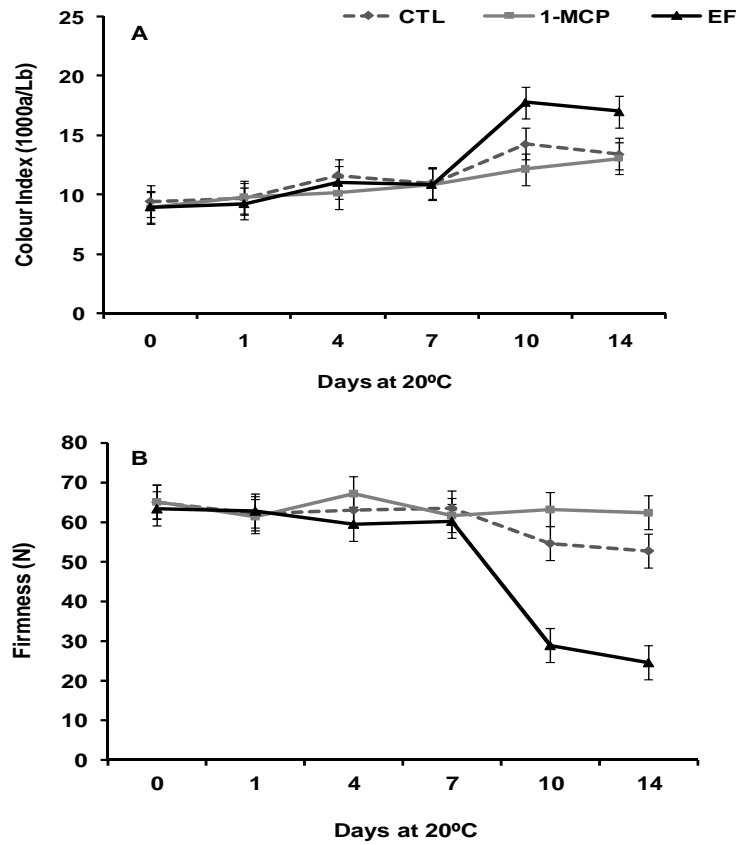


Fig.1. External skin colour (A) and flesh firmness (B) values in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h during 14 days of shelf-life at 20°C. Vertical bars indicate LSD intervals ($P < 0.05$).

Immediately after ethyl formate treatment (day 0), the respiration rate was high compare to non fumigated fruit (Fig. 3A). This may not be surprising, since the ethyl formate treatment used here or as Vapormate™ is applied with CO₂ as the carrier gas. After one day of application, the CO₂ production decreased to show similar levels to other treatments and it remained constant during storage at 20°C. All treatments showed slight changes in ethylene production during the first week of storage. On day 7, the fruit treated with ethyl formate showed a significant increase in ethylene production. Values of increased to 0.011 nmol kg⁻¹ s⁻¹ by day 10 then decreased to values of 0.006 nmol kg⁻¹ s⁻¹ by day 14 (Fig. 3B). Control and 1-MCP treated fruit had ethylene values below 0.0006 nmol kg⁻¹ s⁻¹ throughout the 14 day storage period.

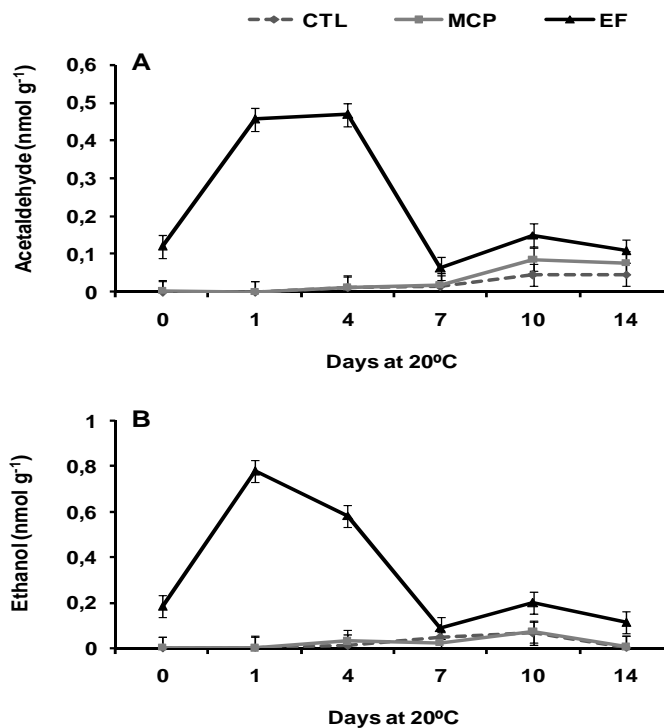


Fig.2. Acetaldehyde (A) and ethanol (B) production in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h during 14 days of shelf-life at 20°C. Vertical bars indicate LSD intervals (P<0.05).

Use of the ETD-300 ethylene system with fruit treated with ethyl formate, and untreated fruit during 14 days of storage, permitted measurement of ethylene at picomole concentrations (Fig. 3C). The evolution of ethylene for both treatments was similar to that observed in the GC method. From day 4 of storage the fruit treated with ethyl formate showed higher ethylene levels ($10 \text{ pmol kg}^{-1} \text{ s}^{-1}$) than control fruit ($5.7 \text{ pmol kg}^{-1} \text{ s}^{-1}$).

Furthermore, fruit treated with ethyl formate showed maximum production levels of ethylene on day 9, reaching values of $285 \text{ pmol kg}^{-1} \text{ s}^{-1}$ and then decreasing to values around $50 \text{ pmol kg}^{-1} \text{ s}^{-1}$ at the end of the storage. Although ethylene production slightly increased from day 9 to the end of the storage in untreated fruit, the ethylene levels were below $50 \text{ pmol kg}^{-1} \text{ s}^{-1}$ for all 14 days of storage. The ethyl formate treated fruit did not soften in the first week of storage where ethylene production was moderate. The increased ethylene production in fruit treated with ethyl formate during days 8 to 14 of storage; coincided with a significant loss of fruit firmness. These results clearly implicate to ethylene as a key factor in the softening of ethyl formate treated fruit.

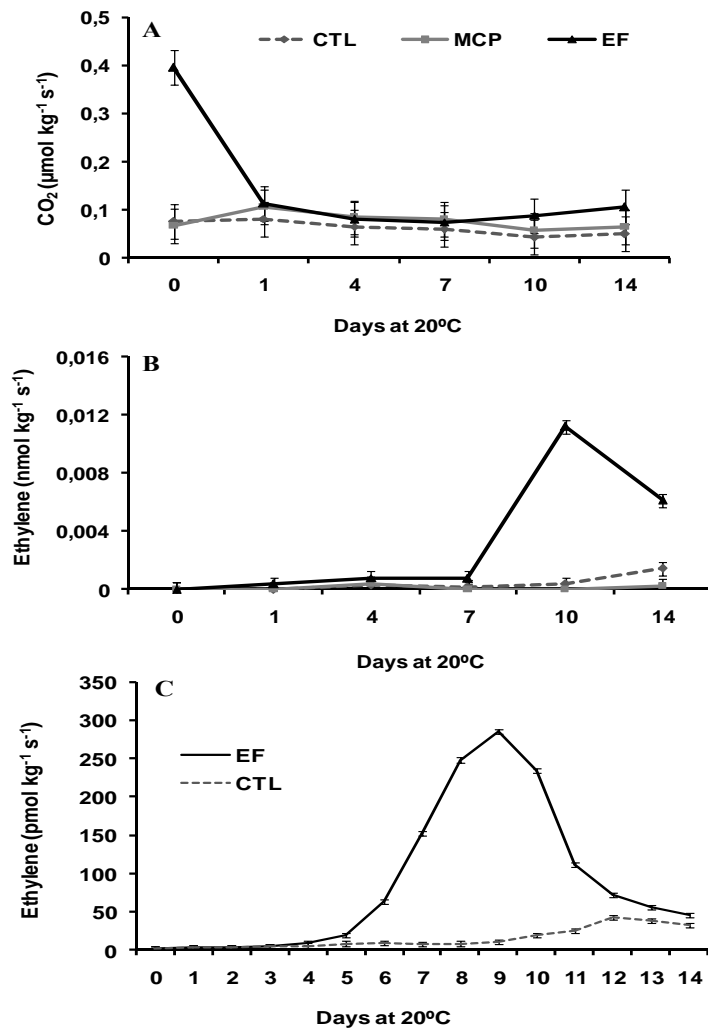


Fig.3. Respiration rate (CO₂) (A) and ethylene (B) production in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h during 14 days of shelf-life at 20°C. Ethylene production from flow through system (C) in persimmon fruit untreated (CTL) and fumigated with 2% ethyl formate (EF) for 4h. Vertical bars indicate LSD intervals (P<0.05).

3.2. Effect of Ethyl Formate on the expression of ethylene and cell wall genes

The relative expression of the ethylene biosynthetic genes *DkACS2*, *DkACO1* and *DkACO2* are shown in Fig.4. 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) are known to be key enzymes in ethylene biosynthesis (Liu et al., 2009). The results from the present study show that the expression of *DkACS2* was higher in both untreated and ethyl formate-treated fruit than in 1-MCP-treated fruit increasing on days 4 and 7 of storage (Fig.4A). The expression in fruit treated with 1-MCP was weak and remained unchanged compared to the values at harvest throughout storage. It is suggested that 1-MCP might restrain the expression of *DkACS2*. The fruit at harvest showed a high relative expression of *DkACO1* that then decreased over storage; the expression was significantly higher in fruit treated with ethyl formate than the other treatments after 7 days of storage (Fig.4B). The expression of *DkACO2* was greatly increased in ethyl formate treated fruit after just 1 day of storage compared to untreated fruit and fruit treated with 1-MCP, which both showed similar values to those observed in fruit at harvest (Fig.4C). As noted above for the expression of *DkACS2*, the 1-MCP treatment suppressed the expression of *DkACO2* and in ethylene biosynthesis. The expression of each isoform of the *DkACO* genes was different and it is possible that each isoenzyme had different factors that induced expression of each gene through differences in transcription levels (Liu et al., 2009b). The increased expression of *DkACO2* at day 1 in fruit treated with ethyl formate occurred at very low ethylene levels while the induction of *DkACO1* and *DkACS2* took place at higher levels of ethylene, indicating that ethylene regulates its own biosynthesis through positive or negative feedback of ACS and ACO (Kende, 1993; Zheng et al., 2006). It was reported recently that ethylene biosynthesis in persimmon fruit is initially induced in the calyx through being modulated by water stress and activation of the expression of *DkACS2* (Nakano et al., 2002). The ethylene produced in the calyx diffuses to the other fruit tissues inducing autocatalytic ethylene production in the fruit by stimulating the expression of *DkACS* and *DkACO* genes (Nakano et al., 2003). In the present research the fruit treated with ethyl formate showed a darkening of the calyx that could be related to the ethylene biosynthesis. Therefore, ethyl formate treatment might be involved in calyx damage and promote the expression of *DkACO1*, *DkACO2* and *DkACS2* consequently to accelerating ethylene biosynthesis.

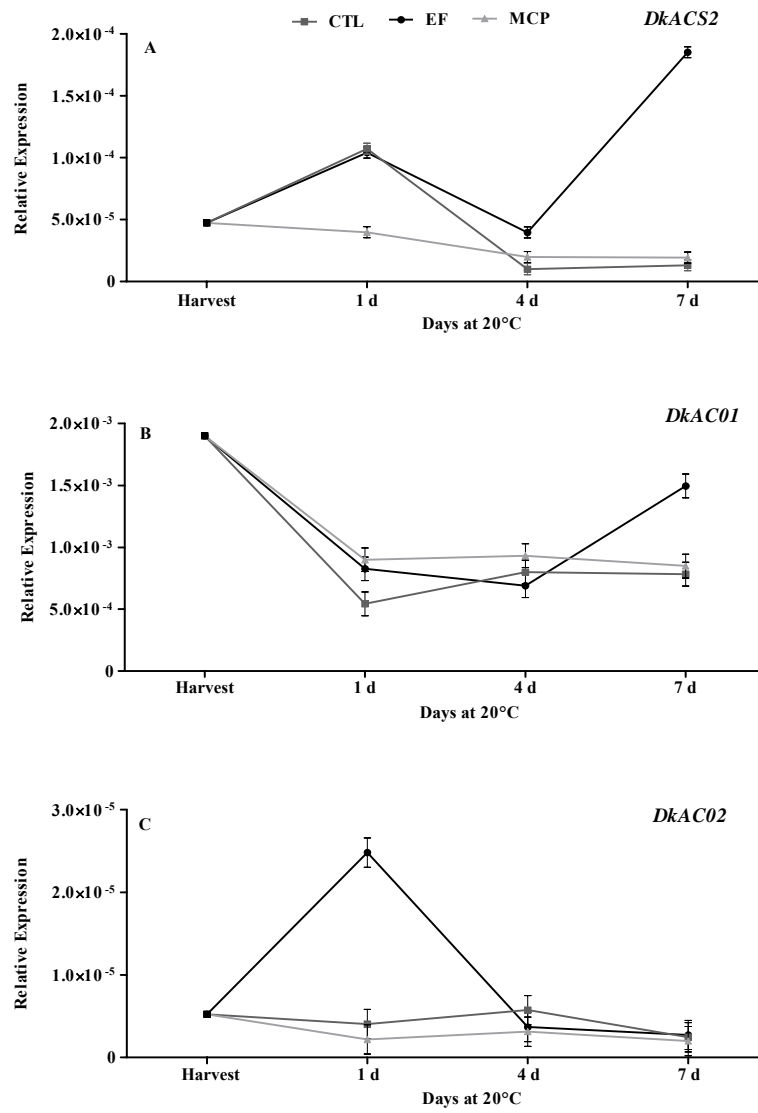


Fig.4. Expression of *DkACS2* (A), *DkACO1* (B) and *DkACO2* (C) genes in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h analyzed at 1, 4 and 7 days of shelf-life at 20°C. Vertical bars indicate LSD intervals (P<0.05).

As well as ethylene production, the ethylene perception also plays an important role in regulating fruit ripening (Pang et al., 2007). The biological effects of ethylene are mediated through a signal transduction pathway in which ethylene receptors are involved (Yin et al., 2012). Fig.5 shows the relative expression of ethylene receptors genes *DkETR1*, *DkETR2* and *DkERS1* in fruit sampled after 1, 4 and 7 days of shelf-life at 20 °C. The expression of *DkETR1* showed no differences between untreated fruit and fruit treated with ethyl formate (Fig.5A). The expression of *DkETR1* in fruit treated with 1-MCP had relative expression below that observed at harvest throughout shelf-life.

Fruit treated with ethyl formate showed higher expression of *DkETR2* than untreated and 1-MCP treated fruit; after 1 day at 20°C; fruit treated with 1-MCP showing the lowest expression of *DkETR2* throughout shelf-life (Fig.5B). This compared with the lower expression of *DkETR1* suggests a greater role for *DkETR2* ethylene biosynthesis in persimmon fruit treated with ethyl formate and 1-MCP.

Expression of *DkERS1*, in fruit treated with ethyl formate showed higher expression than in untreated and 1-MCP treated fruit after one day of fumigation (Fig.5C). After 4 days of storage, the expression of this gene decreased and remained unchanged for the rest of the shelf-life.

It is known that ethylene signalling involves a multi-step pathway in which ethylene receptors act in cascade playing a critical role in regulating ethylene responses (Guo and Ecker, 2004). It has been reported that ethylene treatments applied in Japanese persimmon cv. Hiratanenashi (Pang et al. 2007) clearly increased expression of *DkETR2* and *DkERS1*; however no effect was observed in the level of *DkETR1*; indicating that this gene is ethylene independent. In our experiment the expression from the different ethylene receptors genes of the present study, implied the involvement of *DkETR2* and *DkERS1* in the ethylene metabolism of persimmon fruit. Moreover, the higher expression of these genes in fruit treated with ethyl formate would clearly suggest that ethyl formate treatment may induce the biosynthesis of ethylene receptors genes. The ethylene production seen in fruit treated with ethyl formate (Fig.3B) might promote the expression of *DkETR2* and *DkERS1* genes. The data collected from ethylene production using the ETD-300 ethylene system (Fig.3C) confirmed an increased ethylene production from the beginning of storage in fruit fumigated with ethyl formate. In addition this sharp increase in ethylene production coincided with a physiological response, resulting in a significant decrease in firmness observed

in ethyl formate treated fruit (Fig.1B). It is known that fruit softening is one of the ripening processes that is most sensitive to ethylene. Moreover, it has been shown that ethylene affects the transcription of several ripening-related genes, including those related to softening (Alexander and Grierson, 2002). The role of the ethylene receptor gene *DkERS1* in fruit softening has also been reported by Pang et al. 2007 in which the application of exogenous ethylene to ‘Hiratanenashi’ persimmon led to rapid fruit softening preceded by the induction of the expression of *DkERS1* gene.

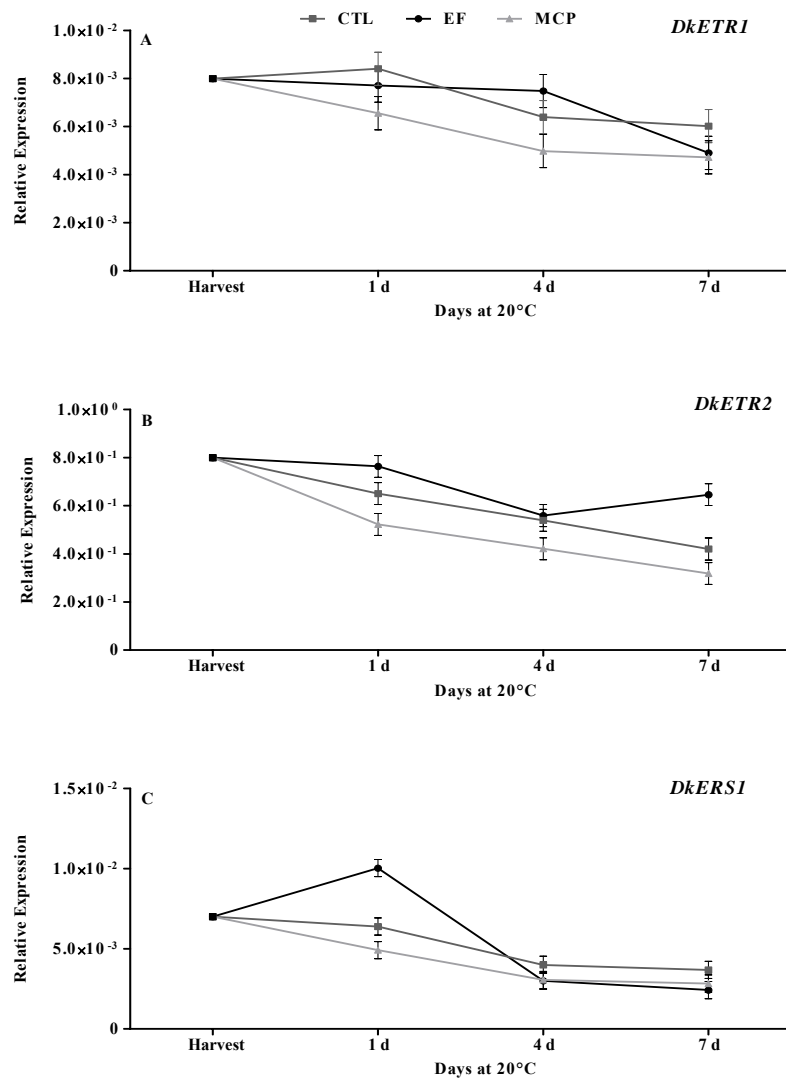


Fig.5. Expression of *DkETR1* (A), *DkETR2* (B) and *DkERS1* (C) genes in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h analyzed at 1, 4 and 7 days of shelf-life at 20°C. Vertical bars indicate LSD intervals (P<0.05)

Fruit softening occurs during ripening as a consequence of progressive cell wall modification and disassembly by enzyme action (Nakatsuka et al., 2011). Members of the *PG*, *XTH* and *EXP* gene families have been shown to be involved in fruit softening processes of persimmons (Nakatsuka et al., 2011; De Souza et al., 2011; Zhu et al., 2013). Fig. 6 shows the relative expression of cell wall ripening genes *DkXTH1*, *DkXTH2*, *DkEXP3*, and *DkPG1*. The relative expression of *DkXTH1* showed no differences among treatments (Fig. 6A); however, the expression of *DkXTH2* showed a higher expression in ethyl formate treated fruit after 1 day of fumigation (Fig. 6B). The expression of these genes in fruit treated with 1-MCP was lower than the other treatments at harvest and throughout storage. This indicates that the expression of both *XTH* genes might be ethylene dependent. Similar results were observed by Zhu et al. 2013 who found that astringent persimmon cv. Fupingjianshi treated with 1-MCP delayed the softening and suppressed the expression of *XTH* genes. Furthermore, an ethylene-dependent response has been observed in *XTH* genes of 'Saijo' persimmon fruit, where *DkXTH1* and *DkXTH2* were suppressed by 1-MCP (Nakatsuka et al., 2011). The relative expression of *DkEXP3* (Fig. 6C) was higher in untreated fruit and fruit treated with ethyl formate than 1-MCP treated fruit which also indicates ethylene dependent activity. Zhang et al. 2012 reported that the expression of *DkEXP3* in 'Fupingjianshi' persimmon was inhibited by exogenous gibberellic acid, a plant growth regulating hormone, which can result in delayed ripening. PG enzymes are known to be necessary for cell division; their activities are known to increase during ripening of fruit, and each enzyme isoform makes a specific contribution to this process (De Souza et al., 2011). The relative expression of *DkPG1* (Fig. 6D) on day 1 was much higher in fruit treated with ethyl formate; it could be a fruit response to ethyl formate treatment, but the decrease of gene expression from harvest to through storage may indicate that *DkPG1* is not the isoform which reveals the drastic softening in 'Fuyu' persimmon. Moreover, Cutillas-Iturralde et al. 1993 reported that polygalacturonase was not apparently involved in the fruit softening process of persimmon fruit ripening after analyzing the polygalacturonase enzyme activity of at different stages of fruit development.

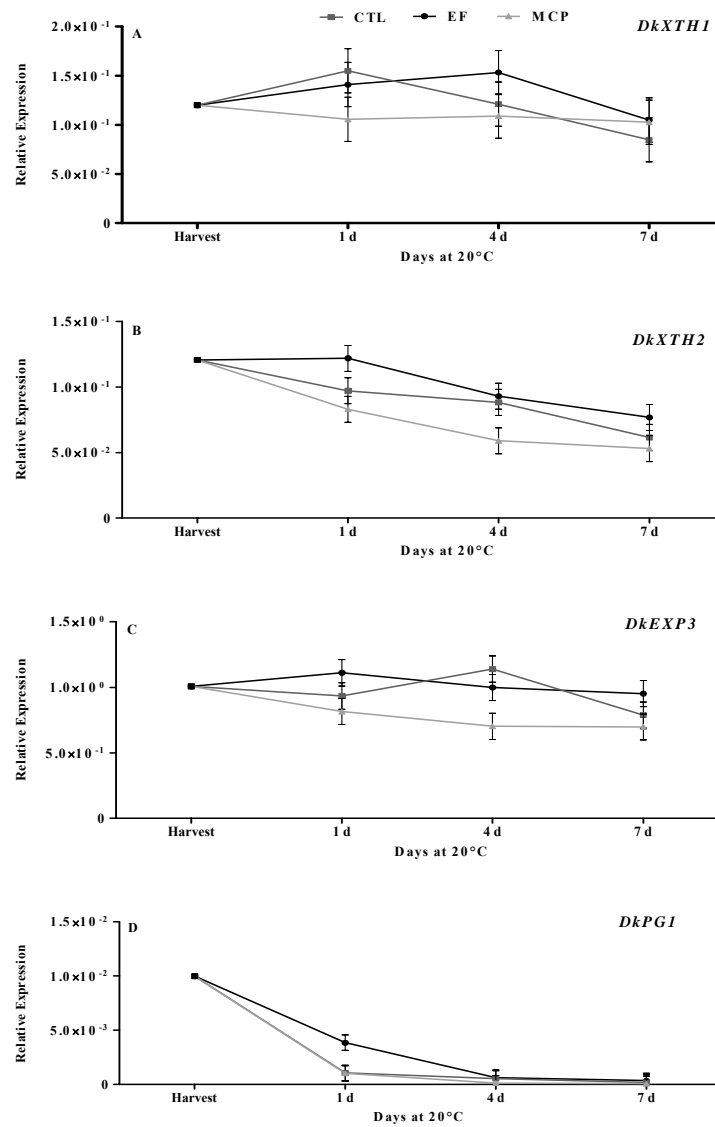


Fig.6. Expression of *DkXTH1* (A), *DkXTH2* (B), *DkEXP3* (C) and *DkPG1*(D) genes in persimmon fruit untreated (CTL), treated with 625 nL L⁻¹ 1-MCP (MCP) or fumigated with 2% ethyl formate (EF) for 4h analyzed at 1, 4 and 7 days of shelf-life at 20°C. Vertical bars indicate LSD intervals (P<0.05)

4. Conclusion

The results presented in this study confirmed the fact that ethyl formate treatment causes significant fruit softening in persimmon fruit. Furthermore, ethyl formate treatment showed a clear effect on ethylene biosynthesis further enhancing the expression of *DkACO1*, *DkACO2*, *DkACS2*, *DkERS1* and *DkETR2* genes with ethylene production increased in the fumigated fruit. 1-MCP treatment significantly inhibited expression of ethylene biosynthesis and ethylene receptors genes. The expression of *DkEXP3*, *DkPG1*, *DkXTH1* and *DkXTH2* did not show evidence of an interaction between ethylene biosynthesis genes and cell wall genes. Since the use of ethyl formate treatment as a fumigant to disinfect fruit and vegetables may involve significant opportunities to meet export and domestic market requirements, further investigation is needed to elucidate the mechanism behind ethyl formate fumigation interacts with ethylene biosynthesis and understanding how this in turn interacts with cell wall genes.

Acknowledgments

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***III.3. PHYSIOLOGICAL AND NUTRITIONAL
STUDY OF PERSIMMON CULTIVARS
INTRODUCED FROM OTHER COUNTRIES
IN ORDER TO BROADEN VARIETAL RANGE***

CHAPTER IX

Fruit quality and response to deastringency treatment of eight persimmon varieties cultivated under Spanish growing conditions

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Abstract

In Spain the persimmon production is based on the cultivar 'Rojo Brillante' which is astringent at harvest (PVA type). The application of postharvest CO₂-treatments that allow removing the astringency while preserving the firmness has been the main factor for the important expansion of persimmon crop in the last decades. Relying the crop only on one variety implied agronomic and commercial risks, thus the availability of new varieties is currently an important research goal. In this sense the Instituto Valenciano de Investigaciones Agrarias (IVIA, Spain) has recently started a program focused on the introduction of varieties from other persimmon growth countries, with positive agronomic features as well as diversity in ripening date, astringency, and fruit characteristics. The study of the postharvest quality of those cultivars with good agronomic performance is necessary for their potential commercial use. In this study the fruit quality at harvest of eight cultivars has been evaluated at two commercial maturity stages; diameter, firmness, external color, total soluble solids and level of astringency were determined. In those cultivars astringent at harvest, the effectiveness of deastringency treatment with high concentration of CO₂, applied in commercial standard conditions, has also been investigated. The analysis of soluble tannins, acetaldehyde production and sensory astringency evaluation revealed that, although this treatment was effective in the most of the cultivars assayed, some of them maintained a level of astringency too high after treatment for consumption. Moreover maturity at harvest influences the effectiveness of the deastringency treatment on some varieties.

Keywords: astringency, soluble tannins, PVA, PCNA, PVNA, PCA.

INTRODUCTION

The 'Rojo Brillante' cultivar currently represents 96% of the total persimmon production in Valencia and 83% of the total Spanish production. A crop based on a monovarietal culture implies several commercial problems and risk that compromise the future of the crop (Naval et al., 2010). In this context the availability of new varieties with positive agronomic features as well as diversity in ripening date, astringency and fruit characteristics would be of great importance.

Fruit astringency is an important attribute of persimmon fruit. Astringency is due to a high concentration of soluble tannins in the flesh that results in an unpalatable sensation of dryness. Persimmon cultivars can be classified into four groups according to the fruit astringency at harvest (Bellini, 1982; Sugiura, 1983; Yonemori et al., 2000): Non-astringent cultivars when seeds are present (Pollination Variant Non Astringent—PVNA); non-astringent cultivars regardless of the presence of seeds (Pollination Constant Non Astringent—PCNA); astringent cultivars when fruit are seedless and mostly astringent when seeds are present (Pollination Variant Astringent—PVA); astringent cultivars regardless of the presence of seeds (Pollination Constant Astringent—PCA).

To be ready for consumption, fruit from the non-PCNA groups (PVNA, PVA and PCA) are commercialized after natural softening/ripening or after removal of astringency in the packing house. An established common practice to remove astringency from astringent fruit is to expose the fruit under anaerobic conditions, at 95–98 % CO₂ for 24–36 h. This treatment allows commercialization of non-astringent fruit with a crispy texture. The effectiveness of the CO₂-treatment to remove astringency is based in the insolubilization of tannins by intermediation of the acetaldehyde generated during anaerobic respiration, which is triggered during exposition of fruit to high-CO₂ atmosphere (Matsuo et al., 1991).

In the present work has been explored the fruit quality at harvest of eight cultivars at two commercial maturity stages and the effectiveness of deastringency treatment with high concentration of CO₂ in those cultivars with astringent fruit at harvest.

MATERIALS AND METHODS

In the present work has been explored the fruit quality at harvest of eight persimmon cultivars: 'Rojo Brillante' and 'Tone Wase' belonging to pollination constant astringent (PVA) group; 'Aizumishirazu-A' and 'Hachiya' belonging to pollination-variant astringent (PCA) group; 'Giboshi' and 'Kaki Tipo' belonging to pollination-variant non-astringent (PVNA) group; 'Jiro' and 'Hana Fuyu' belonging to pollination-constant non-astringent (PCNA) group.

The fruit were harvested from the persimmon germplasm collection hosted by the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain) at two harvest date: 18 October (H1), when 'Rojo Brillante' achieved commercial external color (homogeneous orange tones) and 9 November (H2).

Ten seedless fruits of each variety from two trees were picked in each harvest date. Moreover additionally ten fruits of non-PCNA groups were harvested in order to submit them to deastringency treatment under standard conditions for 'Rojo Brillante' persimmon (95% CO₂ at 20°C and 90% R.H, during 24h).

Just after harvest, and after two days of deastringency treatment, measurement of color, firmness, equatorial diameter, soluble tannins (ST) and acetaldehyde (AcH) production were carried out. Besides astringency were evaluated by sensorial analysis.

External color was determined over 10 fruits using a Minolta colorimeter (Model CR-300 Ramsey, NY, USA). Hunter parameters 'L', 'a', 'b', were measured and results were expressed as color index: CI=1000a/Lb.

Flesh firmness was evaluated at harvest with a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA) using an 8-mm plunger. Fruit firmness values were an average of 10 fruits per treatment. Results were expressed as load in Newtons (N) to break the flesh in each fruit on 180° sides after peel removal.

To determinate soluble tannins and acetaldehyde concentration, lots of 6 fruit per treatment were divided into three samples (3 replicates; 2 fruit per replicate) and cut into four longitudinal parts. Two opposite parts were sliced and frozen at -20 °C to determine soluble tannins. The other opposite parts of the fruit were placed in an electric juice extractor and the filtered juice was then

used to determine acetaldehyde concentration. Soluble tannins were evaluated using the Folin-Denis method (Taira, 1995), the results were expressed in percentage of fresh weight. Acetaldehyde concentration was measured in two replicates per juice sample and analyzed by headspace gas chromatography as described by Salvador et al. (2004); results were expressed as mg/100mL.

The sensory evaluation of astringency was performed on composite samples of five (peeled and sliced) fruit from each replicate. A trained panel of 8–10 people familiar with persimmon fruit was asked to evaluate astringency. A four-point scale was used, where 0 was no astringency and 3 was very high astringency. Samples were presented to members of the panel in trays labeled with random three-digit codes and served at room temperature ($25 \pm 1^\circ\text{C}$). The judges had to taste several segments of each sample in order to compensate, as far as possible, for the biological variation of the material. Milk was provided for palate rinsing between samples.

The data were subjected to the analysis of variance, and multiple comparisons between means were determined by the least significant difference test ($P= 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD).

RESULTS AND DISCUSSION

In Mediterranean area the harvest season of ‘Rojo Brillante’ cultivar takes place between October and the end of December. External color is usually the harvest maturity index for this cultivar; when the skin of the fruit acquires homogeneous orange tones the fruit is considered as commercial. Nevertheless this cultivar attains reddish-tones at the end of the season (Salvador et al., 2007).

In this study the first harvest (H1) was carried out when ‘Rojo Brillante’ persimmon achieved external homogeneous color (Color Index (CI)=7.3 and firmness=53N). Three weeks later, other harvest (H2) was performed, when the fruit of this cultivar showed higher coloration (CI=15.6 and firmness=42.5N) (Fig. 1A and 1B).

At both harvest dates, among all studied cultivars, 'Tone Wase' persimmon exhibited the greatest external coloration ($CI > 20$). Nevertheless a point to take into account is that the flesh firmness of the fruit was very low, 13.8N and 7.6N in H1 and H2 respectively. The firmness is an important quality parameter of the persimmon for commercialization. Nowadays there is not a quality standard for this parameter but for 'Rojo Brillante' persimmon, values of firmness of 10N are considered the commercial limit (Salvador et al. 2004). According with previous studies 'Rojo Brillante', maintains higher firmness even when fruit is colored dark red at the end of the season (Salvador et al., 2007).

'Hachiya' and 'Giboshi' cultivars also exhibited higher CI than 'Rojo Brillante' and adequate fruit firmness values for commercialization at both evaluated harvest dates. The fruit from Aizumishirazu-A cultivar, at H1 showed CI values of 10.4, slightly higher than 'Rojo Brillante'; nevertheless at H2, meanwhile fruit from 'Rojo Brillante' increase significantly the external coloration, this increase was lower in 'Aizumishirazu-A', maintaining orange tones. 'Kaki Tipo', is the cultivar with greater delay in the maturation, showing a pale orange color with slightly green areas; this is reflected in the low values of CI at both harvest dates.

The two non-astringent cultivars, 'Jiro' and 'Hana Fuyu', showed low external color at H1, nevertheless when harvested later displayed attractive tones (CI close to 16), similar to 'Rojo Brillante'.

All cultivars presented a high fruit size, and the color increase observed in the second harvest was accompanied by an increase in size in all cultivars (Fig. 1C). 'Rojo Brillante' and 'Hana Fuyu' were the cultivars with the largest fruit.

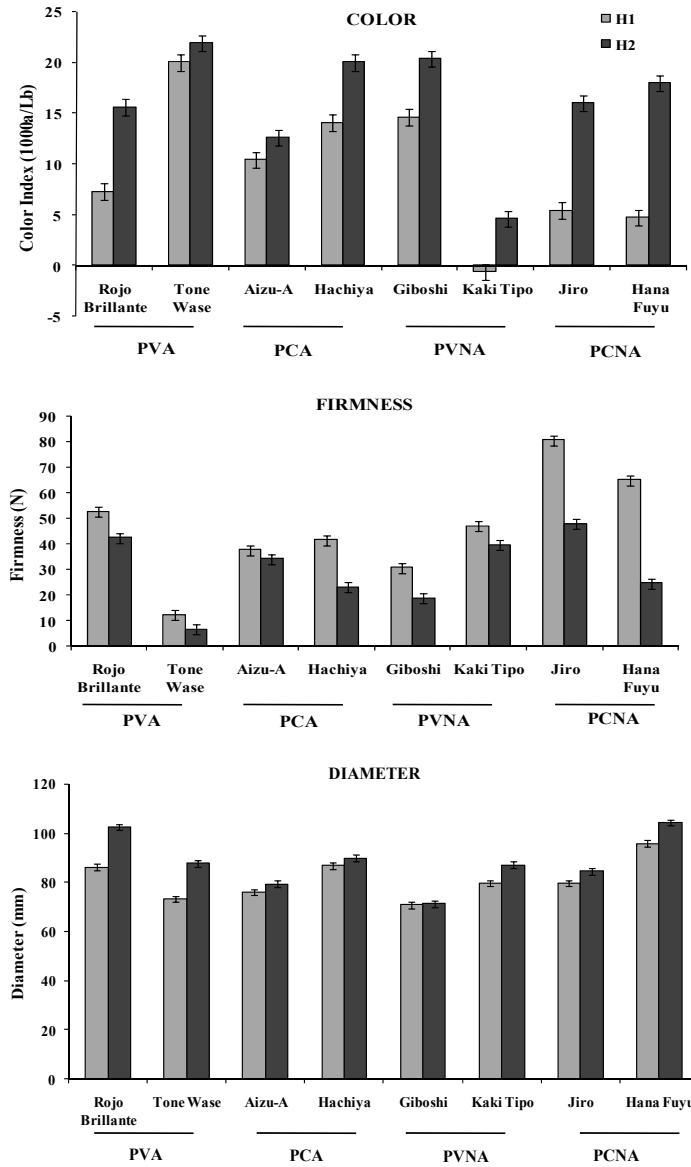


Fig.1. External color (Color index (CI)=1000a/Lb) (A) , Firmness (Newton) (B), Diameter (mm) (C) of fruit from eight persimmon varieties, at two harvest date: (H1) 18 October and (H2) 9 November. Vertical bars represent LSD intervals (P=0.05). **PCA** (*Pollination-Constant and Astringent*); **PVA** (*Pollination-Variant and Astringent*); **PVNA** (*Pollination-Variant and Non Astringent*); **PCNA** (*Pollination-Constant and Non Astringent*)

Regarding the astringency of the fruit at harvest, the sensory evaluation showed that the fruit from PCNA cultivars exhibited absence of astringency with values of soluble tannins (ST) lower than 0.09% at two harvest dates (data not shown); nevertheless the fruit from the PVA, PCA and PVNA groups were classified as very astringent, exhibiting values of ST between 0.4% to 1% depending to the cultivars (Table1).

The loss of astringency based on the fruit exposure under anaerobic conditions, is due to the increase of acetaldehyde production generated in the anaerobic respiration. Soluble tannins responsible for astringency are polymerized by the acetaldehyde produced to form insoluble compounds, which are non-astringent (Taira et al., 1997). It has been reported that soluble tannin content below 0.1% in persimmon fruit does not produce astringency sensation (Vidrih et al., 1994). This threshold has been assumed later in several studies (Yamada et al., 2002). However in some varieties of persimmon soluble tannin content as low as 0.06% can produce a detectable astringency (Besada et al., 2010).

In this work, the fruit from all cultivars after deastringency treatment suffered an important decrease in the soluble tannins content respect the values at harvest; this drop in ST content was accompanied with an increase of the acetaldehyde production (Table 1).

After treatment, the fruit from 'Rojo Brillante', cv. 'Giboshi' and 'Kaki Tipo' showed values of soluble tannin between 0.03- 0.04%, and were sensory evaluated as non-astringent. The fruit from 'Aizumishirazu-A', with values of 0.06% ST was also evaluated as non-astringent.

'Tone Wase' persimmons at H1 showed values of ST (0.34%); after deastringency treatment a significant reduction was observed and fruit presented absence of astringency. A point to take into account is that when fruit were harvested later (H2), with lower ST (0.15%), the treatment was not completely effective, fruit being evaluated with moderate astringency. This loss of efficacy has been associated to the low flesh firmness of the fruit at H2 (7.5N). In previous studies a lack of efficacy in the deastringency treatment has been reported when it is applied to fruit with low values of fruit firmness (Salvador et al., 2008).

At both harvest dates, fruit from 'Hachiya' were evaluated with 'moderate' or 'high' astringency, so the deastringency treatment was not effective in this variety under the studied conditions.

In general, the deastringency treatment caused loss of firmness in all studied varieties; the highest firmness loss was observed in fruit from 'Hachiya' and 'Tone Wase'. In previous studies have been reported that postharvest application of CO₂-enriched atmospheres leads to deterioration of cellular membranes, which may cause a decrease on firmness. Moreover the mechanical effect caused by insolubilized tannins on tannin cells membranes has been also linked with this decreased on firmness (Satoshi et al., 1998; Salvador et al., 2007).

Table 1. Firmness (N), soluble tannin content (% fw), acetaldehyde concentration (mL/100mL) and sensory evaluation of astringency of fruit from different persimmon varieties of PCA, PVA and PVNA group, at harvest time and after CO₂ treatment, at two harvest date: (H1) 18 October and (H2) 9 November.

Group/Variety	Firmness (N)		Soluble tannin content (%)		Acetaldehyde (mg/100mL)		Sensory Astringency		
	Harvest	After CO ₂	Harvest	After CO ₂	Harvest	After CO ₂	Harvest	After CO ₂	
<u>PCA</u> Aizumishirazu A	H1	38.2 a	37.2 a	0.93 a	0.060 c	0.37 b	3.18 a	High	Absence
	H2	33.7 a	34.6 a	0.60 b	0.060 c	0.51 b	3.08 a	High	Absence
Hachiya	H1	43.6 a	39.5 b	0.74 a	0.190 c	0.14 b	1.19 a	High	High
	H2	26.9 c	19.2 d	0.31 b	0.050 d	0.36 b	1.23 a	High	Moderate
<u>PVA</u> Tone wase	H1	13.8 a	10.7 a	0.34 a	0.017 d	0.28 c	1.24 b	High	Absence
	H2	7.6 ab	4.9 b	0.15 b	0.050 c	0.21 c	2.26 a	High	Moderate
Rojo Brillante	H1	53.3 a	52.0 c	0.20 a	0.037 c	0.22 c	2.97 a	High	Absence
	H2	43.8 b	41.2 b	0.18 a	0.030 c	0.26 c	2.39 b	High	Absence
<u>PVNA</u> Giboshi	H1	30.9 a	30.5 a	0.57 a	0.034 c	0.28 b	2.55 a	High	Absence
	H2	20.4 ab	17.3 b	0.27 b	0.037 c	0.48 b	2.18 a	High	Absence
Kaki tipo	H1	51.2 ab	43.1 ab	0.25 a	0.040 b	0.20 c	3.99 a	High	Absence
	H2	40.5 ab	38.9 b	0.34 a	0.030 b	0.33 c	2.19 b	High	Absence

Different letters for each parameter and cultivar indicate significant differences (95% LSD-test)

CONCLUSIONS

According to the results obtained in this study, all evaluated cultivars could have a commercial interest. The varieties Jiro and Hana Fuyu, from PCNA group, showed absence of astringency at both harvest dates, so these varieties could be commercialized without deastringency treatment.

The applied deastringency treatment was completely effective in ‘Rojo Brillante’, ‘Aizumishirazu-A’, ‘Giboshi’ and ‘Kaki Tipo’. Therefore these varieties could be good alternative to expand the varietal range. The deastringency treatment under the assayed conditions was not effective in fruit from ‘Hachiya’; so in this case, it would be necessary to optimize the conditions of the process.

The interest of ‘Tone Wase’ is due to its earlier harvest compared to ‘Rojo Brillante’ persimmon, nevertheless a point to take into account is that the effectiveness of the deastringency treatment depends on the fruit firmness at harvest.

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Chapter IX

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CHAPTER X

Influence of persimmon astringency type on physico-chemical changes from the green stage to commercial harvest

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Abstract

Persimmon (*Diospyros kaki*, Lf.) cultivars are currently classified into four astringency types based on two significant characteristics, astringency of fruit at harvest and variable pollination (PCNA, PVNA, PCA, PVA). The aim of this study was to evaluate the influence of astringency type on physicochemical changes occurring during persimmon maturation. To this end, unseeded fruits of 10 persimmon cultivars of four persimmon types were evaluated in three maturity stages according to skin colour: green, colour-breaking and orange-reddish. The Principal Component Analysis showed that soluble tannins content, total antioxidant capacity, total soluble solids and the accumulation pattern of individual sugars were affected by astringency type. During maturation, all the cultivars exhibited a decline of firmness, and also increased flesh carotenoids and acetaldehyde production. Such changes were cultivar-dependent rather than type-dependent. All the cultivars also shared a similar CO₂ production trend.

Keywords: Persimmon, Maturation, Astringency type, Soluble tannins, Sucrose, Glucose, Fructose, Total antioxidant capacity, Carotenoids

1. Introduction

Persimmon (*Diospyros kaki* Lf.) belongs to the *Diospyros* L. genus (Ebenaceae), and is believed to have originated in China, from there it was widely distributed worldwide from tropical to temperate regions with suitable climatic conditions, such as Brazil, Italy, Spain, Israel, Iran, New Zealand or Australia (FAOSTAT, 2008; Mowat et al., 1995). The persimmon cultivars exhibit a singular characteristic that is not common to other fruit trees; thus fruits from some persimmon cultivars are astringent at harvest, while others produce non-astringent fruits. Accordingly, persimmon cultivars are classified into two astringency categories, non-astringent and astringent cultivars, depending on the concentration of the soluble tannins present in pulp when fruits are harvested. Based on two significant persimmon characteristics (fruit astringency at harvest and variable pollination), persimmon cultivars are currently classified into four astringency types: Pollination Variant Non-Astringent (PVNA) (non-astringent cultivars when seeds are present); Pollination Constant Non-Astringent (PCNA) (non-astringent cultivars, regardless of the presence of seeds); Pollination Variant Astringent (PVA) (astringent cultivars when fruit are seedless and mostly astringent when seeds are present); Pollination Constant Astringent (PCA) (astringent cultivars, regardless of the presence of seeds). ‘Variant pollination’ is expressed as the browning of the tissues surrounding locules when seeds are present, whereas flesh colour type is invariable with the ‘pollination constant’.

As with most fruits, knowledge of the physicochemical changes associated with persimmon maturation is essential to not only determine the optimum maturity stage for harvesting, but also as a base to study postharvest behaviour. The response of persimmon fruits to different postharvest treatments has been shown to rely heavily on the fruit maturity stage at harvest (Besada et al., 2008 and 2010a). In line with this, some physicochemical changes, other than the evolution of tannins that take place during the persimmon maturation process have been evaluated in specific cultivars, such as cv. Rojo Brillante, cv. Kaki Tipo or cv. Harbiye (Candir et al., 2009; Del Bubba et al., 2009; Salvador et al., 2007). These studies, which have related the evolution of external colour fruit (the non-destructive index currently used for harvesting persimmon fruit) with the internal changes during maturity, have provided useful information about these particular cultivars.

Nowadays, the impact of astringency type on changes in tannins during maturation is well-known. Yet very little information is available about the

influence of astringency type on the evolution of other relevant physicochemical parameters during the persimmon maturity process, especially for unseeded fruits, which are not affected by pollination type. Similarly after reviewing knowledge on persimmon composition, Giordani et al., (2011) claimed that the ripening stage of samples is of paramount importance in comparative studies among cultivars.

In this context, this study aimed to investigate the physicochemical changes associated with the maturity process of 10 persimmon cultivars that belong to the four persimmon types (PVNA, PCNA, PVA and PCA). To this end, the main physicochemical parameters were evaluated in unseeded fruits in three maturity stages: green coloured fruit, colour break stage and fruit exhibiting the orange-reddish commercial colour. A principal components analysis, an analysis of variance and a study of the correlations between parameters were performed to obtain as much information as possible from the data.

2. Materials and methods

2.1. Plant material and experimental design

The following cultivars were studied in the present work: ‘Rojo Brillante’ and ‘Tonewase’ (PVA-type cultivars); ‘Aizumishirazu-A’, ‘Giombo’ and ‘Hachiya’ (PCA-type cultivars); ‘Giboshi’ and ‘Kaki Tipo’ (PVNA-type cultivars); ‘Jiro’, ‘O’goshō’ and ‘Hana Fuyu’ (PCNA-type cultivars). They all form part of the persimmon germplasm collection hosted by the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia), Spain.

From August to December (2012), 30 unseeded fruits from each cultivar were harvested in three different maturity stages (maturity stage 1 (SI): fruit with a homogenous green skin; maturity stage 2 (SII): colour-break stage, fruits showing a yellowish-green skin; maturity stage 3 (SIII): commercial maturity, fruits showing no visible green background, but the characteristic orange or orange-reddish colour of each cultivar). After harvest, fruits were taken to the IVIA, where they were carefully evaluated in order to perform three homogeneous replicates of seven fruits free of damage for each cultivar and maturity stage. Then measurements of external colour, firmness, carbon dioxide and ethylene, and total soluble solids, were taken. Besides, fruit samples were

frozen to determine acetaldehyde and ethanol production, soluble tannin content, total and individual sugars, total antioxidant capacity and total carotenoids.

2.2. Evaluation of the physicochemical parameters

External colour was determined in a Minolta colorimeter (Model CR-300 Ramsey, NY, USA) on three replicates of seven fruits each by taking two measurements on opposite equatorial areas on each fruit. Hunter parameters 'L', 'a', 'b', were measured and the results were expressed as Colour Index=1000a/Lb, which accurately reflect the skin colour changes of persimmon fruits (Salvador et al., 2007). Respiration rate (CO₂ production) and ethylene production were measured with three replicates of individual fruits, which were incubated in hermetic 1-litre jars for 2 h at 20°C. One ml of air from the headspace was withdrawn with a hypodermic syringe and injected into a Perkin Elmer gas chromatograph equipped with a Poropak QS 80/100 column. A thermal conductivity detector was used to determine carbon dioxide. Helium was the carrier gas used at 9.2 psi. The injector, oven and detector temperatures were 115°C, 35°C and 150°C, respectively. A flame ionization detector was used to determine ethylene. Helium was the carrier gas at 8 psi. Injector, oven, and detector temperatures were 175, 75 and 175°C, respectively; CO₂ production was expressed as ml CO₂ kg⁻¹ h⁻¹ and ethylene production as µl C₂H₄ kg⁻¹ h⁻¹.

Flesh firmness was evaluated at harvest by a Texturometer Instron Universal Machine (model 4301, instron Corp., Canton, MA, USA) using an 8-mm plunger. The results were expressed as load in Newtons (N) to break the flesh in each fruit on 180° sides after removing peel (3 replicates of 6 fruits each).

Immediately after measuring firmness, each fruit was cut into four longitudinal parts. Two opposite parts were placed into an electric juice extractor and filtered juice was used to determine total soluble solids (TSS) and the acetaldehyde (AcH) concentration (3 lots of juices, each from 6 fruits). TSS were evaluated using a digital refractometer (Atagomod. PR1) and the results were expressed in °Brix. The AcH concentration was analysed by headspace gas chromatography, as described by Salvador et al. (2004), and the results were expressed as mg/100mL.

The other two opposite fruit parts were sliced and frozen with liquid N₂ to be ground and kept at -80°C to evaluate the following parameters: soluble tannins, antioxidant capacity, total carotenoids and sugars.

Soluble tannins were evaluated by the Folin-Denis method described by Taira (1995), modified by Arnal et al. (2004). The results were expressed as a percentage of fresh weight (fw).

Antioxidant capacity was determined as the antiradical activity of methanolic extracts, where 20 mg of dried sample were homogenised with 2 ml of methanol (80% v/v). It was spectrophotometrically tested by measuring absorbance at 515 nm of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), adapted from Novillo et al. (2014). The obtained values were compared to the concentration-response curve of the standard Trolox solution, expressed as micromoles of Trolox equivalents (TE) per 100 mg of dry weight (dw).

Total carotenoid content was determined according to Koca et al. (2007), where 50 mg of dried sample were homogenised with 2 ml of a hexane:acetone mixture (7:3). Carotenoid content was determined spectrophotometrically at 450 nm as β -carotene equivalents (β CE) from the standard curve.

Extraction and determination of sugars were conducted from 50 mg of dried sample, extracted with 1 ml of twice-distilled water. The extracted sample was centrifuged twice at 14,000 rpm for 15 min at 4°C. The supernatant was filtered through 0.45 μ m filters and purified through a Sep-Pak C18 column. The sugars analysis was performed on a Thermo Separation Products HPLC. Separation of sugars was performed isocratically with Millipore water as the mobile phase, which flowed at 0.5 mL min⁻¹ in a fast carbohydrate column (Aminex HPX87-C column), 100 x 7.8 mm i.d. (Bio Rad Laboratories, Hercules, CA, USA), preceded by a micro-guard cartridge, Carbo-C 30 x 4.6 mm i.d. (Bio Rad Laboratories, Hercules, CA, USA), maintained at 75°C and attached to a refractive index detector (RID). Concentrations were calculated with the corresponding external standards and expressed as μ g/mg dw.

The following standards were used to determine sugars, carotenoids and antioxidant capacity: sucrose, fructose and glucose; β -carotene and trolox solution from Sigma-Aldrich Chemie (Steinheim, Germany).

2.3. Statistical analysis

A principal components analysis (PCA), an analysis of variance (ANOVA) and a study of the correlations between parameters were performed.

The ANOVA was carried out using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA), and multiple comparisons were made between means by the least significant difference test ($P \leq 0.05$).

A PCA was run and included the normalised data (log 2-transformed) from the 10 studied cultivars, with the following parameters: firmness, acetaldehyde, carbon dioxide production, soluble tannins content, total antioxidant capacity, concentrations of fructose, sucrose and glucose, total soluble solids and total carotenoids. Ethylene production was not included because it remained at undetectable levels for all the cultivars and maturity stages. The Acuity 4.0 program (Axon Instruments) was used with distance measures based on Pearson's correlation to perform both the PCA and the study of correlations.

3. Results and discussion

3.1. External colour

Evolution of external colour from green to green-yellow, and later to orange or orange-redish tones, depending on the cultivar, is the most obviously change during persimmon maturation (Zhou et al., 2011). Figure 1 present the skin colour index exhibited by the 10 selected cultivars in the three maturity stages under study: completely green fruits (SI), fruits in the colour-break stage (SII) and fruits with the homogeneous commercial colour.

In some cultivars, such as 'Tonewase', 'Rojo Brillante', 'Kaki Tipo' and 'O'gosho', the change noted from green to oranges tones occurs homogeneously all around the fruit and therefore, persimmons are considered to obtain the commercial colour (stage SIII) when showing homogeneous orange tones (colour index value close to +10). However in other cultivars ('Aizumishirazu-A', 'Giombo', 'Hachiya', 'Jiro', 'Hana Fuyu' and 'Giboshi') colour change is irregular and most of the surface depict reddish-orange tones

when the green tones are been lost. Thus the color index at commercial maturity (SIII) is high (close to +20).

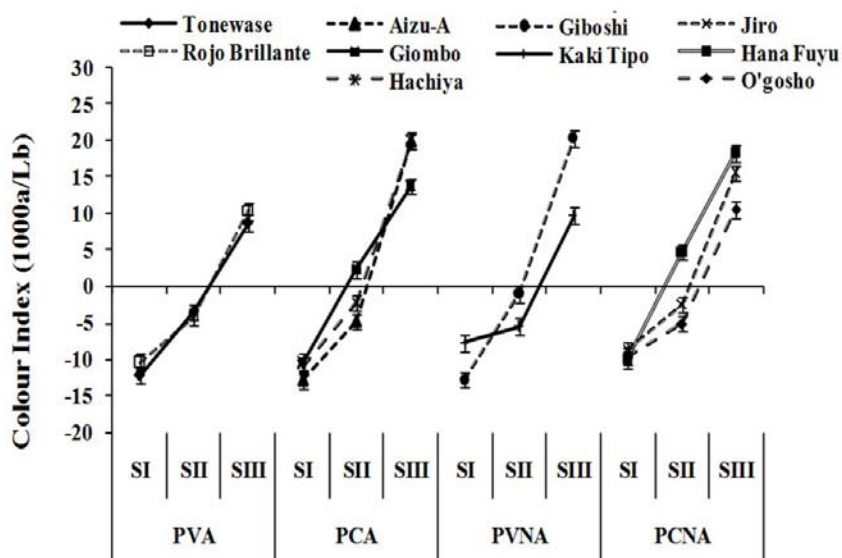


Fig. 1. Evolution of external colour (1000a/Lb) during the maturation of ten persimmon cultivars. Three maturity stages were evaluated: SI- green stage; SII- colour-break; SIII-characteristic homogeneous orange or orange-reddish tones. Vertical bars represent LSD-intervals when comparing all the cultivars and maturity stages ($P < 0.05$).

3.2. Soluble tannin content and total antioxidant capacity, the main parameters that differentiate the maturation of astringent and non-astringent cultivars

The Principal Components Analysis, which was done using the complete data set (all the cultivars, maturity stages and physicochemical parameters), revealed that 82.2% of total variance was explained by the first two principal components (Fig. 2). However, the third component, that which explained 7.8% of variance, clearly separated the persimmon samples into two groups (Fig. 2A): a first group of fruits from naturally non-astringent cultivars (PCNA-type

cultivars: ‘Jiro’, ‘O’goshō’ and ‘Hana Fuyu’); a second group of cultivars that were astringent at harvest, which includes the PCA- (‘Aizumishirazu-A’, ‘Giombo’ and ‘Hachiya’), PVA- (‘Tonewase’ and ‘Rojo Brillante’) and PVNA-type (‘Giboshi’ and ‘Kaki Tipo’) cultivars. In both these groups (non-astringent and astringent), the first and second components, which explained most variability, were associated with maturity stage. So the samples in maturity stage SI were located in the right and upper spaces in relation to those in stage SIII (located to the left and downwards). Stage SII was located in the middle of stages SI and SIII.

The loading plots revealed the main parameters responsible for separation between the groups (astringent and non-astringent cultivars; Fig. 2B). As expected, soluble tannins (ST) content was the most relevant parameter for separating these two cultivar groups. Besides soluble tannins, total antioxidant capacity greatly contributed to differentiate them. Hence at the three evaluated maturity stages, the level of both ST and total antioxidant capacity was higher in the astringent cultivars compared with the non-astringent ones. According to Yonemori and Matsushima (1985, 1987), tannin cell development in astringent cultivars continues until late fruit growth stages, while tannin cell development stops in early fruit growing stages in non-astringent cultivars (PCNA-type). Early cessation of tannin cell development is thought to be the main cause of natural astringency loss in PCNA-type fruits as it results in a diluted tannins concentration in flesh as fruits grow. The proximity to the location of soluble tannins content and antioxidant capacity in the loading plot space can be explained by the high radical scavenging activity that soluble tannins possess (Gu et al., 2008). In fact a strong positive correlation between soluble tannins content and total antioxidant capacity was detected ($r=0.93$).

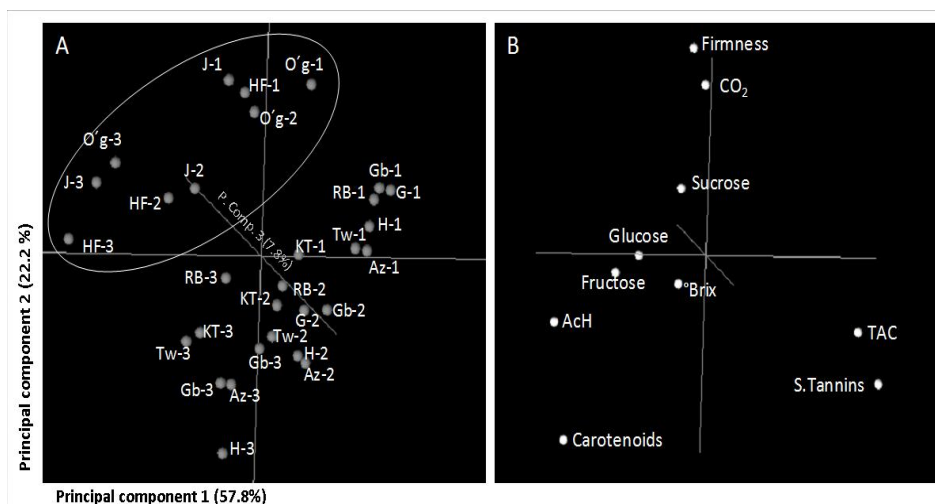


Fig. 2. Principal component analysis of the physicochemical parameters of persimmon fruits of the total data set (10 cvs. and 3 maturity stages). The score and loading plots for the first, second and third principal components are shown. Three replicates were analysed per cultivar in each maturity stage; the mean values of the replicates are shown. (*RB*: Rojo Brillante; *Tw*: Tonewase; *Az*: Aizumishirazu-A; *Gb*: Giombo; *H*: Hachiya; *G*: Giboshi; *KT*: Kaki Tipo; *J*: Jiro; *O'g*: O'goshō' and *HF*: Hana Fuyu. The number after the initials indicates the stage of maturity).

The ANOVA corroborated the importance of these two factors for differentiating the astringent cultivars from the non-astringent ones (Fig 3A and 3B). In both cultivar groups, a decline in soluble tannins content with advanced maturation was observed (Fig. 3A). Yet while the soluble tannins content ranged from 0.6% in SI to 0.05-0.03% in SIII in the non-astringent cultivars, the tannins concentration in the astringent cultivars was higher and ranged from 2.3% in SI to 0.5-1% in SIII (Fig. 3A). Thus in the three studied maturity stages, the soluble tannins concentration was clearly higher in the astringent *vs.* non-astringent cultivars. The antioxidant capacity analysis revealed a similar trend to that observed for soluble tannins content (Fig. 3B). Antioxidant capacity decreased in all the cultivars and in parallel to the decline in soluble tannins associated with maturation. The antioxidant capacity of the astringent cultivars ranged from 130 $\mu\text{mol TE}/100\text{mg}$ in SI to 20 $\mu\text{mol TE}/100\text{mg}$ in SIII, while these values ranged from 40 $\mu\text{mol TE}/100\text{mg}$ to 10 $\mu\text{mol TE}/100\text{mg}$ for the non-astringent group.

It is worth highlighting that in both groups (astringent and non-astringent cvs.) relevant differences in the profile of soluble tannins and total antioxidant capacity were detected among cultivars (Fig. 3A and 3B). In maturity stage SI, cultivars 'Tonewase', 'Aizumishirazu-A' and 'Giombo' obtained an ST concentration close to 2.5%, while the other astringent cultivars displayed a significantly lower content, around 1.5%. Significant differences among cultivars were also observed in the commercial maturity stage (SIII) since 'Rojo Brillante' exhibited a 0.4% content, while that of 'Aizumishirazu-A' came close to 1%. Among the non-astringent cultivars, the main differences in the ST concentration were observed in SI, when cv. O'gosho showed a 2-fold higher soluble tannins content (0.6%) than cultivars 'Fuyu' and 'Jiro' (0.3%). Yet in maturity stage SIII, all the non-astringent cultivars gave an ST concentration value close to 0.06%.

Our results for antioxidant capacity (Fig. 3B) showed that despite its high correlation with ST content, other compounds must provide antioxidant capacity to persimmon fruits. This fact became particularly clear when comparing cultivars 'Giboshi' and 'Kaki Tipo' since they showed the same ST content throughout maturation. However, the total antioxidant capacity of 'Kaki Tipo' was significant lower than that of 'Giboshi', and was also lower than that of all the other cultivars.

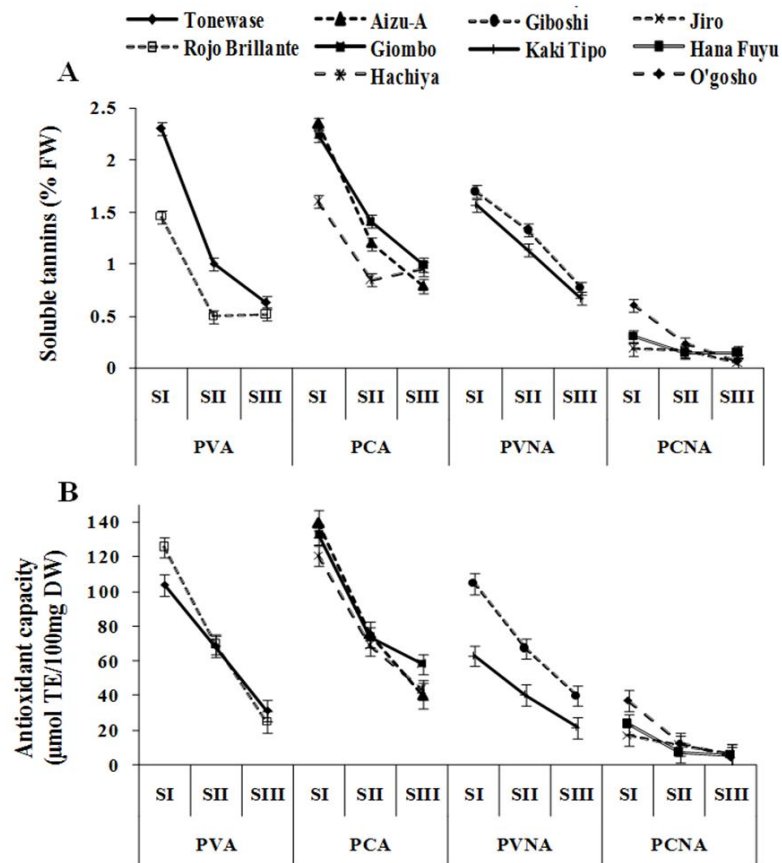


Fig. 3. Evolution of soluble tannin content and total antioxidant capacity during the maturation of ten persimmon cultivars. Three maturity stages were evaluated: SI-green stage; SII-colour-break; SIII-characteristic homogeneous orange or orange-reddish tones. Vertical bars represent LSD-intervals when comparing all the cultivars and maturity stages ($P<0.05$).

3.3. Evolution of the physicochemical parameters not responsible for astringency during the maturation process

In order to further explore the differences among maturity stages, Principal Components Analyses were performed independently for the non-astringent (Fig. 4A and Fig. 4B) and astringent cultivars (Fig. 4C and Fig. 4D). In both cultivar groups, this analysis allowed us to group samples according to the maturity stage of fruits.

The samples from the non-astringent cultivar group were separated according to maturity stage by the first two principal components (Fig. 4A). Component 1 (79% of variance) separated SI and SII (located on the right) from SIII (located on the left), while component 2 (11% of variance) separated SII (located in the upper space) from SI and SIII, situated at the bottom of the space.

With the astringent cultivars (Fig. 4C), the three maturity stages were separated by the component 1, which explained 60% of variability. SI was located on the right, SII in the central space and SIII on the left of the space. However, one exception was observed since the fruits of cultivar ‘Kaki Tipo’ in stage SI were closer to the samples from stage SII of the other cultivars (in the central area of the space) than to those in stage SI. It is also worth mentioning that the cultivar ‘Tonewase’ samples were separated from the other cultivars by component 2, while the samples of this cultivar in the three maturity stages were located at the bottom of the space.

The observation made of the loading plot of both the non-astringent and astringent cultivar groups (Fig. 4B and 4D) revealed that the concentrations of acetaldehyde and carotenoids were the parameters that helped separate the samples in stage SIII from those in earlier maturity stages. Thus the highest levels of both acetaldehyde and carotenoids were associated with the most advanced maturity stage (SIII). Meanwhile, the earliest maturity stage (SI) was characterised by showing, besides the highest levels of soluble tannins and antioxidant capacity, the greatest firmness and the highest CO₂ production values.

The lowest antioxidant capacity level, shown by ‘Kaki Tipo’ if compared to the other above-mentioned astringent cultivars (Fig. 3B), explains why its samples in stage I are located in the PCA space close to the samples in stage II

of the other cultivars. Thus the 'Kaki Tipo' fruits in stage I showed a similar antioxidant capacity level (60 $\mu\text{mol TE}/100 \text{ mg}$) to that shown by the other astringent cultivars in stage II (Fig. 3B).

As regards the particular location of cultivar 'Tonewase', according to the loading plot (Fig. 4D), the separation of this cultivar from the other astringent ones for all three maturity stages took place because it had a higher content of carotenoids and sugars.

In order to further understand the changes associated with the maturation of the different cultivars, an ANOVA was performed for each individual parameter.

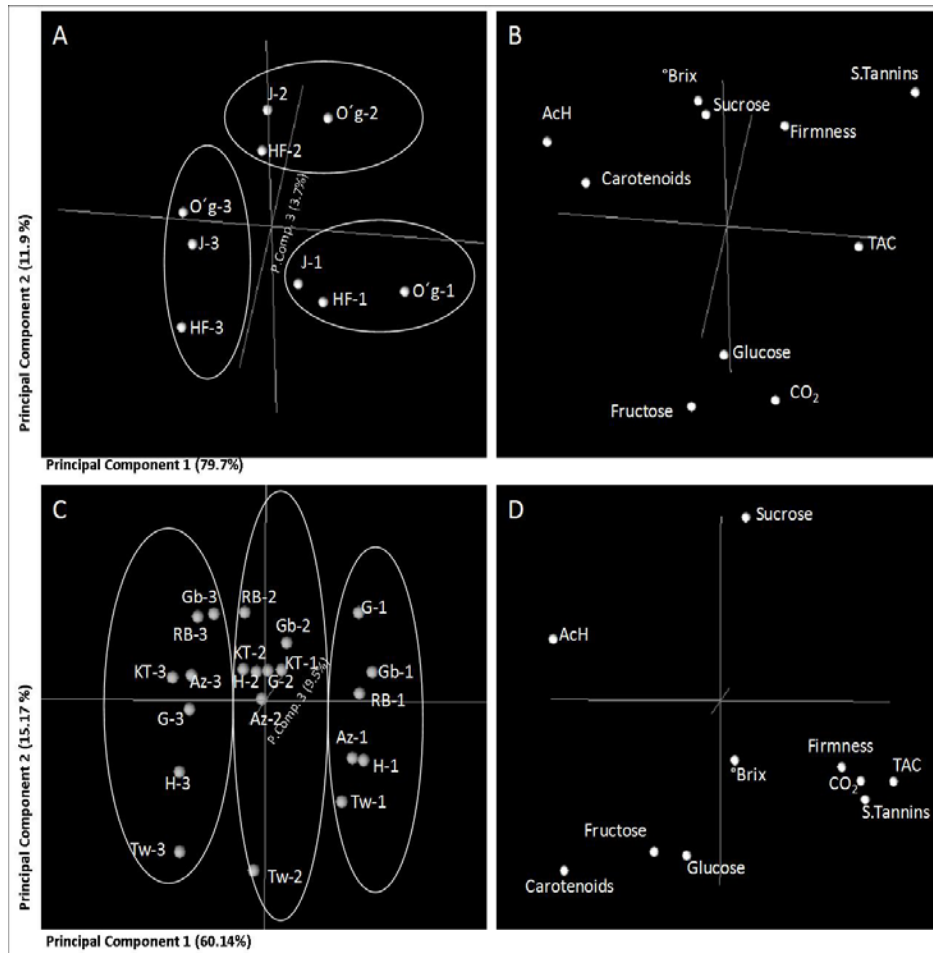


Fig. 4. Principal component analysis of the physiological and physicochemical parameters of persimmon fruits. The score and loading plots for the first, second and third principal components are shown. a, b Data set (PCNA-cultivars, three maturity stages). c, d Data set (PCA, PVA and PVNA-cultivars, three maturity stages). Three replicates were analysed per cultivar in each maturity stage; the mean values of the replicates are shown. (*RB*: Rojo Brillante; *Tw*: Tonewase; *Az*: Aizumishirazu-A; *Gb*: Giombo; *H*: Hachiya; *G*: Giboshi; *KT*: Kaki Tipo; *J*: Jiro; *O'g*: O'gosho' and *HF*: Hana Fuyu. The number after the initials indicates the stage of maturity).

3.3.1. *Flesh carotenoids accumulation and softening during maturation are affected mainly by the specific cultivar*

Similarly to the changes in skin colour exhibited by persimmon fruits during maturation, flesh colour also evolves from white to orange tones. Like that observed in loquat cultivars, in which red-fleshed and white-fleshed fruits can be distinguished, some persimmon cultivars depicted a much more intense orange coloured flesh upon commercial harvest than others (Zhou et al., 2011). Carotenoids compounds are known to be mainly responsible for the flesh colour of persimmons (Ebert et al., 1985; Yuang et al., 2006), and β -Cryptoxanthin and Zeaxanthin have been reported as the most abundant carotenoids (Zhou et al, 2011).

In the present study, the 10 evaluated cultivars gradually accumulated carotenoids in flesh during maturation (Fig. 5). For all the cultivars, the largest increment of carotenoid content was observed between stages SII and SIII; that is, from the colour-break stage to the stage when fruits exhibited a homogeneous external colour. This suggests that accumulation of carotenoids in flesh and skin may occur in parallel. However, the relationship between internal and external colours was cultivar-dependent. ‘Tonewase’ and ‘Rojo Brillante’ showed not significant differences in external colour throughout maturation (Fig. 1), but carotenoids accumulated considerably more in the flesh of cultivar ‘Tonewase’ (75 β CE μ g/100 mg) than in ‘Rojo Brillante’ (20 β CE μ g/100 mg) (Fig. 5). Such differences in carotenoids accumulation were related to the visual flesh colour. Thus cultivars ‘Rojo Brillante’, ‘Giombo’ and ‘O’goshu’, whose flesh was the lightest in colour, showed a 2-fold lower total carotenoids content (20-40 μ g/100 mg) than the other cultivars (80-120 μ g/100 mg).

An astringent effect category was observed for fruit firmness in the earliest maturity stage (SI) as all the non-astringent cultivars presented greater firmness (close to 100N) than the astringent ones (close to 80N) (data not shown). Yet these differences disappeared as the maturity process advanced. A gradual decline in firmness was noted in all the cultivars associated with fruit maturation, which was characteristic for each cultivar. In stage III, firmness values differed, and ranged from 47N (cv. Giombo) to 14N (cv. Tonewase) among the astringent cultivars. Similarly the firmness values of the non-astringent cultivars fell between 65N (cv. O’Goshu) and 24N (cv. Fuyu).

Therefore intensity of softening, which took place during maturation, was associated with neither astringency category nor astringency type, but was

cultivar-dependent. The study of the correlations found between external colour and firmness revealed a strong negative correlation ($r=-0.9$) between these two parameters in most cultivars, excluding cultivar 'Tonewase', which showed a poorer correlation ($r=-0.6$). The 'Tonewase' fruits did not exhibit any significant decrease in firmness in relation to external colour evolution between stages SI and SII. Nevertheless, a major drop in the firmness values was recorded between maturity stages II and III. Loss of firmness that persimmon fruits underwent during maturation has been associated by Salvador et al. (2007) to microstructural changes in flesh. A progressive degradation of the parenchyma, which gradually shows less swollen and more deformed cells, leads to generalised intercellular adhesion loss.

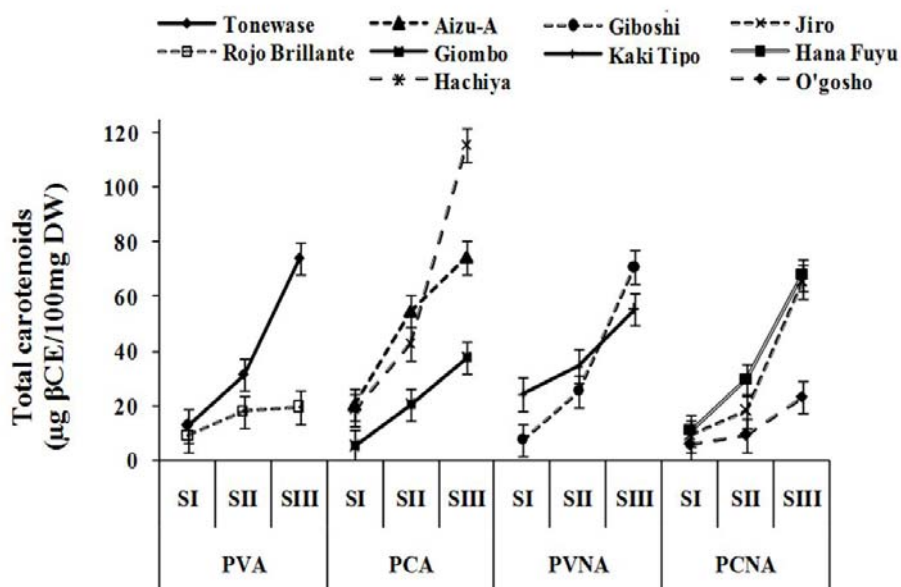


Fig. 5. Evolution of the total carotenoids flesh content during the maturation of ten persimmon cultivars. Three maturity stages were evaluated: SI- green stage; SII-colour-break; SIII-characteristic homogeneous orange or orange-reddish tones. Vertical bars represent LSD-intervals when comparing all the cultivars and maturity stages ($P<0.05$).

3.3.2. All the studied cultivars share a similar pattern of CO₂, ethylene and acetaldehyde production

All the studied cultivars showed a similar pattern of CO₂ production (data not shown). The highest values were observed in the green maturity stage (SI), when both the astringent and non-astringent cultivars gave CO₂ values which ranged from 9 to 15 ml kg⁻¹ h⁻¹. Upon colour-break (SII), production gave lower values within the 2-4 ml kg⁻¹ h⁻¹ range, which then slightly increased to values of around 4-8 ml kg⁻¹ h⁻¹ when fruits presented a homogeneous colour (SIII).

Our study revealed that ethylene production remained at undetectable levels in all the cultivars throughout the three studied maturity stages (data no shown). Although persimmon fruits are considered climacteric fruit (Tanaka, 1983), it is well-known that they produce very low levels of ethylene, and are highly sensitive to the presence of exogenous ethylene (Besada et al., 2010b; Salvador et al., 2007).

A common pattern was observed for acetaldehyde production in the four persimmon types. Thus a gradual increase in acetaldehyde production was associated with maturation in all the cultivars. In fact a positive correlation ($r=0.79$) was observed between skin colour evolution and acetaldehyde production. Note that this study was performed with seedless fruits, so acetaldehyde production was unrelated to presence of fruit seeds. All the cultivars showed acetaldehyde production to be lower than 1 mg/100 mL in the commercial maturity stage (data no shown).

3.3.3. Total soluble solids and sugars accumulation seem influenced by astringency type

Table 1 shows the total soluble solids (TSS) and the content of individual sugars for each cultivar and maturity stage. TSS, expressed as °Brix, increased in all the cultivars as maturation progressed, although a persimmon category effect was seen as the PCNA-type cultivars obtained lower TSS values (8.2-15.2 °Bx) than the astringent types (PCA, PVA, PVNA) (13.5-19.6 °Bx) for all three maturity stages. It is noteworthy that the TSS content in persimmon fruit includes not only sugars, but also soluble tannins. So this parameter could reflect both an increase in sugars and a decrease in soluble tannins, which happen throughout maturation. Therefore the TSS measurement could be considered a good indicator of fruit maturity in non-astringent cultivars (Ullio, 2003) since the level of soluble tannins was very low in the maturity stages,

which came close to the commercial stage and did not interfere with TSS measurements. Yet for the astringent cultivars, in which soluble tannin contents remained high, TSS had to be ruled out as an indicator of fruit sweetness.

The main sugars found in persimmon fruit flesh were sucrose, glucose and fructose (Table 1), which agrees with the work by Del Bubba et al. (2009). The non-astringent cultivars (PCNA-type) shared a similar sugar accumulation profile, which differentiated them from the astringent ones. In the PCNA-type cultivars, the concentrations of glucose and fructose lowered from SI to SII, and then increased again in SIII, while sucrose concentrations remained at relatively constant values. In commercial maturity stage SIII, sucrose was the major sugar in cv. 'Jiro' and 'O'gosho' (310.8 and 224.8 $\mu\text{g}/\text{mg}$, respectively), while glucose and fructose were the main sugars in cv. Hana Fuyu (295.9 and 277.2 $\mu\text{g}/\text{mg}$, respectively).

Different sugar evolution trends were observed among the astringent cultivars. The glucose and fructose content of cultivars 'Tonewase', 'Hachiya', 'Giboshi' and 'Kaki Tipo' rose as maturation evolved, while sucrose content exhibited slight changes (Table 1). This trend was similar to that reported by Del Bubba et al. (2009), who observed increasing glucose and fructose contents and an increasing-decreasing parabolic-like sucrose concentration. It must be noted the sugars content observed in cultivar 'Tonewase' had the highest contents of glucose (from 269.6 in SI to 509.9 $\mu\text{g}/\text{mg}$ in SIII) and fructose (from 198.1 in SI to 412.1 $\mu\text{g}/\text{mg}$ in SIII), along with the lowest sucrose values (116.6 in SI, 38.3 at SII and 74.7 $\mu\text{g}/\text{mg}$ in SIII) throughout maturation; this fact explains the separation of this cultivar from the other astringent cultivars in the Principal Components Analysis space (Fig. 4C). It must also be mentioned that cultivar 'Kaki Tipo' fruits stand out for their high and similar content of all three sugars in the commercial stage (sucrose: 356.5, glucose: 394.4, fructose: 335.7 $\mu\text{g}/\text{mg}$).

The second trend was observed in cultivars 'Aizumihirazu-A', 'Rojo Brillante' and 'Giombo', which showed increased sucrose, fructose and glucose throughout maturation; this pattern agrees with that observed by Senter et al. (1991) and Zheng and Sugiura (1990), who all reported rising sucrose and reducing sugars. Among these cultivars, 'Rojo Brillante' obtained the highest accumulation of sugars in stage SIII.

Giordani et al. (2011) attributed the different trends they observed in sugars accumulation to invertase enzyme activity, which suggests that this enzyme activity may be influenced by soluble tannins content. Moreover, inhibition of invertase activity by gallic and tannin acid has been reported by Chen et al., (2003). The results reported herein revealed that the non-astringent cultivars showed similarly low and slightly changed levels of soluble tannins throughout maturation, while the astringent cultivars displayed a highly marked changing level of ST during this process. These tannins evolution trends noted in the two astringency cultivar categories may be key to explain how PCNA-cultivars shared similar invertase activity and, therefore, the same sugars accumulation pattern, while depending on the specific cultivar in the astringent group. Besides, the enzymatic systems responsible for glucose production in persimmon fruits, such as gluconeogenesis pathway enzymes, fructose isomerase and cellulose, have been reported to also be key factors for free sugars composition (Ittah 1993).

Table 1. Total soluble solids and individual sugar concentrations of ten persimmon cultivars in three maturity stages. SI-green stage, SII-colour-break and SIII-characteristic homogeneous orange or orange-reddish tones. Different letters in the same column indicate significant differences among the cultivars in each maturity stage. Different letters (capital letters) in the same row indicate significant differences among the maturity stages for each cultivar. (LSD test, $P < 0.05$)

GROUP Cultivar	TSS (^o Brix)			Sucrose ($\mu\text{g}/\text{mg dw}$)			Glucose ($\mu\text{g}/\text{mg dw}$)			Fructose ($\mu\text{g}/\text{mg dw}$)		
	SI	SII	SIII	SI	SII	SIII	SI	SII	SIII	SI	SII	SIII
PVA												
Tonewase	17.3h ^A	18.7f ^B	18.9d ^B	116.6a ^B	38.3a ^A	74.7a ^{AB}	269.6f ^A	268.7e ^A	509.9g ^B	198.1e ^A	206.3e ^A	412.1g ^B
RojoBrillante	13.5d ^A	16.5c ^B	17.5c ^C	290.4e ^A	357.0e ^B	396.9e ^B	203.3de ^A	203.2d ^A	317.7e ^B	140.7cd ^A	162.1de ^A	264.0de ^B
PCA												
Aizu-A	16.6f ^A	18.7f ^B	19.6e ^C	153.9b ^A	236.4bcd ^B	241.2bcd ^B	180.9bcd ^A	139.9abc ^A	194.5bc ^B	116.3bc ^A	104.6bc ^A	171.9bc ^B
Giombo	16.0e ^A	16.6c ^B	17.7c ^C	199.6cd ^A	248.7bcd ^A	401.9e ^B	169.2bc ^A	131.4ab ^A	240.2cde ^B	124.8bcd ^A	96.4ab ^A	201.7bcd ^B
Hachiya	16.6f ^A	17.7d ^B	17.9c ^B	195.3bc ^{AB}	277.1cd ^B	147.6ab ^A	197.3de ^{AB}	153.7abcd ^A	281.5de ^B	98.1b ^A	105.1bc ^A	232.6cde ^B
PVNA												
Giboshi	16.8fg ^A	18.4f ^B	19.1de ^C	238.0d ^A	203.6bc ^A	143.3ab ^A	114.5a ^A	167.6bcd ^A	204.3bcd ^B	64.1a ^A	125.4bcd ^B	183.7bc ^B
Kaki Tipo	17.2gh ^A	18.6f ^B	19.0de ^B	298.4e ^{AB}	204.8bc ^A	356.5de ^B	193.7cde ^A	198.6cd ^A	394.4f ^B	150.9d ^A	147.8cd ^A	335.7f ^B
PCNA												
Jiro	9.7c ^A	12.9b ^B	15.2b ^C	299.7e ^A	284.5de ^A	310.8cde ^A	164.4b ^B	121.3ab ^A	152.2ab ^{AB}	131.7cd ^B	99.9ab ^A	146.5ab ^B
Hana Fuyu	8.2a ^A	12.5b ^B	13.3a ^C	200.1cd ^A	185.5b ^A	199.0b ^A	196.3cde ^B	133.9ab ^A	295.9c ^B	142.7cd ^B	102.8abc ^A	277.2ef ^C
O'goshio	9.0b ^A	11.3a ^B	13.5a ^C	182.6bc ^A	237.7bcd ^A	224.8bc ^A	209.3e ^C	99.4a ^A	120.8a ^B	143.8cd ^C	55.6a ^A	97.9a ^B

4. Conclusion

This study of physicochemical changes associated with the maturity process of 10 persimmon cultivars belonging to the four persimmon types (PVNA, PCNA, PVA and PCA) revealed that astringency type does not determine the decline in flesh firmness, carotenoids accumulation and acetaldehyde production, which occur in parallel with external colour evolution, and that these changes are cultivar-dependent. Nevertheless, astringency type clearly influenced not only soluble tannins content and antioxidant capacity, but also the accumulation of individual sugars. Therefore, despite all the cultivars presenting a declining antioxidant capacity through maturation, which has been related with a drop in soluble tannins, both these parameters were higher in the astringent cultivars in all the maturity stages. Moreover, the non astringent-cultivars shared a common sugar accumulation pattern, while different trends were observed in the astringent cultivars. All the cultivars shared a similar CO₂ production pattern.

Acknowledgments

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CHAPTER XI

Nutritional composition of ten persimmon cultivars in the 'ready-to-eat crisp' stage. Effect of destringency treatment

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Abstract

Traditionally persimmons have been consumed over-ripened to avoid astringency perception. However, the introduction of new technology that removes astringency while preserving fruit firmness has allowed the commercialization of 'ready-to-eat crisp' fruits. Several studies have evaluated the nutritional composition of over-ripened persimmons and have claimed that they are a good source of primary and secondary metabolites that are favourable for human health. Yet very little is known about the nutritional composition of persimmons in the 'ready-to-eat crisp' stage. In this context, we determined the main nutritional compounds in ten popular persimmon cultivars, including astringent cultivars ('Rojo Brillante', 'Tone Wase', 'Giboshi', 'Kaki Tipo', 'Aizumishirazu-A', 'Giombo', 'Hachiya') and non-astringent cultivars ('O'goshi', 'Hana Fuyu' and 'Jiro'). To this end, fruits were harvested when their texture was firm, and soluble polyphenols content, total antioxidant capacity and main sugars, organic acids and carotenoids were evaluated. In those astringent cultivars at harvest, the changes in nutritional compounds associated with applying deastringency treatment with high CO₂ concentration were determined. Our results revealed the main sugars (glucose, fructose and sucrose), organic acids (citric acid, malic acid and succinic) and carotenoids (β -cryptoxanthin, lutein, violoxanthin, zeaxanthin, and β -carotene) present in the flesh of crisp persimmons. At harvest the content of these metabolites vastly varied among cultivars; astringent cultivars showed higher soluble polyphenols and greater antioxidant capacity, and presented higher contents of sugars and organic acids than non-astringent ones. The deastringency treatment applied to astringent cultivars resulted in a drastic loss of soluble polyphenols and total antioxidant capacity, and induced changes in carotenoids and sugars composition.

Keywords: Persimmon, Crispy texture, Nutritional Composition, Antioxidant Capacity

1. Introduction

An important feature of persimmon cultivars (*Diospyros kaki* L.) is that some produce astringent fruits due to the high soluble tannin content in flesh. Astringency is the sensation that results when soluble tannins bind salivary proteins and cause them to precipitate or aggregate, which leads to a rough "sandpapery" or dry sensation in the mouth. According to the level of flesh soluble tannins at harvest, persimmon cultivars can be classified into two general categories: astringent and non-astringent persimmons (also called 'sweet' persimmons) [1].

Astringent cultivars, unlike non-astringent ones, require postharvest treatment to remove astringency prior to commercialising fruits. Traditionally, fruits from astringent cultivars were consumed in the over-ripening stage, which can be achieved naturally on trees or by applying exogenous ethylene. Apart from loss of astringency in both cases, fruits suffer significant flesh softening, which makes postharvest handling difficult. In recent decades, several deastringency techniques have been assayed to remove astringency while preserving the firm texture of fruits. Nowadays, the most widely used technique to remove fruit astringency without it affecting firmness is based on exposing fruits to anaerobic conditions (95-98 % CO₂ for 24-36 h), which results in ready-to-eat non-astringent fruits with a crispy texture.

Due to health awareness campaigns, the general public has become more interested in foods that support and promote health. Thus the importance of fruit bioactive metabolites as protective compounds in human nutrition and health is well recognised. Indeed persimmons are considered a good source of readily available carbohydrates and bioactive compounds, like polyphenols and carotenoids [2,3]. Studies *in vivo* and *in vitro* have suggested a relevant role of persimmon extracts in cell protection against free radicals to prevent damage to important biological membranes [4-6].

Despite several research works having studied the chemical composition of different persimmon cultivars [3,7,8], it is difficult to compare the data obtained in the aforementioned studies because, besides the cultivar, many other factors, e.g. maturity stage, environmental factors and technological process, affect the content of the primary and secondary metabolites [3,9,10]. Most data refer to over-ripened fruit and it is necessary to know the chemical composition of 'ready-to-eat crisp' fruits, as mentioned previously by Giordani *et al.* [3].

Unfortunately, very little information about the effect of destringency treatment on phytonutrients is available.

Therefore, the aim of this research was to study the nutritional composition of ten persimmon cultivars in the 'ready-to-eat crisp' stage by establishing the main primary and secondary metabolites, such as sugars, organic acids, carotenoids and soluble polyphenols. The effect of destringency treatment on nutritional composition was also evaluated in astringent cultivars.

2. Material and methods

2.1. Vegetal material and experimental design

Persimmon fruits from seven astringent cultivars (cv. Rojo Brillante; cv. Tone Wase; cv. Giboshi; cv. Kaki Tipo; cv. Aizumishirazu-A; cv. Giombo; cv. Hachiya) and three non-astringent cultivars (cv. O'goshō; cv. Hana Fuyu; cv. Jiro) were harvested from October to November in the persimmon germplasm collection hosted by the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Harvesting was carried out when fruits reached a homogeneous full orange or reddish-orange colour depending on the cultivar. In all the cultivars, fruits presented a crisp texture with firmness values over 30 N.

One homogeneous lot of 20 persimmon fruits from each cultivar was evaluated immediately after harvest. A second lot of 20 fruits from astringent cultivars was subjected to destringency treatment under standard conditions (95 % CO₂ at 20 °C and 90 % RH for 24 h). After destringency treatment, fruits were kept at room temperature in an air atmosphere for 24 h to evaluate the changes associated with treatment application.

At harvest and after destringency treatment (with astringent cultivars), besides measuring external colour and firmness, the following parameters were evaluated: soluble polyphenol content, total antioxidant capacity, total soluble solids, total and individual sugars, organic acids and carotenoids compounds. The astringency level was sensory-evaluated by a semi-trained panel. With astringent cultivars, acetaldehyde concentration was analysed at harvest and after destringency treatment.

2.2. Fruit assessments

2.2.1. Evaluation of external skin colour, flesh firmness, acetaldehyde production and sensory analysis

External skin colour and flesh firmness were determined on 20 fruits. External colour was evaluated with a Minolta colorimeter (Model CR-300 Ramsey, NY, USA); Hunter parameters ('L', 'a', 'b') were measured and the results were expressed as a colour index: $CI=1000a/Lb$ [11]. Fruit firmness was determined by a Texturometer Instron Universal Machine model 4301 (Instron Corp., Canton, MA, USA) using an 8-mm plunger and breaking the flesh in each fruit on 180° sides after removing peel.

Acetaldehyde concentration was analysed by headspace gas chromatography, as described by Salvador *et al.* [11] and the results were expressed as $\text{mg } 100 \text{ mL}^{-1}$.

The sensory evaluation of astringency was performed on composite samples of three (peeled and sliced) fruits. A semi-trained panel of 8-10 people, who were familiar with persimmon fruits, was asked to evaluate astringency. A 4-point scale was used, where 0 was "no astringency" and 3 was "very high astringency". Samples were presented to panel members on trays labelled with random 3-digit codes and served at room temperature ($25 \pm 1 \text{ }^\circ\text{C}$). Judges had to taste several segments of each sample to compensate, as far as possible, the biological variation of the material. Milk was provided for palate rinsing between samples.

2.2.2. Determination of soluble polyphenol content and total antioxidant capacity

The soluble polyphenol content of each cultivar was measured as gallic acid equivalents (GAE) using the Folin-Ciocalteu's phenol reagent (FC reagent) according to Taira [12]. The results were expressed as milligrams of GAE per 100 g of dried weight (dw).

Total antioxidant capacity was determined as the antiradical activity of methanolic extracts, where 20 mg of sample were homogenised with 2 mL of methanol (80 % v/v). It was spectrophotometrically tested by measuring absorbance at 515 nm of free radical DPPH, adapted from Novillo *et al.* [13].

The obtained values were compared to the concentration-response curve of the standard Trolox solution expressed as micromoles of Trolox equivalents (TE) per 100 mg of dw.

2.2.3. Extraction and HPLC analysis of sugars and organic acids

The extraction and determination of sugars and organic acids were conducted from 50 mg of sample and extracted with 1 mL of bi-distilled water. The extracted sample was centrifuged twice at 14,000 rpm for 15 min at 4 °C. The supernatant was filtered through 0.45 µm filters and purified in a Sep-Pak C18 column. An analysis of sugars was performed in a Thermo Separation Products HPLC. Separation of sugars was done isocratically with Millipore water as a mobile phase, at a flow of 0.5 mL min⁻¹ in a fast carbohydrate column (Aminex HPX87-C column), 100 x 7.8 mm i.d. (Bio Rad Laboratories, Hercules, CA, USA), which was preceded by a micro-guard cartridge, Carbo-C 30 x 4.6 mm i.d. (Bio Rad Laboratories, Hercules, CA, USA) maintained at 75 °C and attached to a refractive index detector (RID). Separation of the organic acids analysis was carried out in an Alliance liquid chromatographic system (Waters, Barcelona, Spain) equipped with a 2695 separation module, coupled to a 2996 photodiode array detector and a ZQ2000 mass detector. A thermostat column oven, an ICsep ICE-COREGEL 87H3 column (Transgenomic), an ICsep ICE-COREGEL 87H guard kit and an automatic injector were used for chromatographic separation. The Empower 2 software was used for data acquisition. Sample temperature was 5°C and column temperature was 35 °C. Capillary voltage was 3.0 kV, cone voltage was 23 V, source temperature was 100 °C, desolvation temperature was 200 °C and desolvation gas flow was 400 L/Hr. Full data acquisition was performed by scanning 100 to 400 uma in the centroid mode. The solvent system was an isocratic mobile phase of 0.1 % H₂SO₄ solution. The total run time was 20 min at 0.6 mL min⁻¹. The injection volume was 5 µL. The results were expressed as µg mg⁻¹ dw. Sucrose, glucose and fructose sugars were identified by comparing their retention time with a standard and they were quantified using an external calibration curve. Organic acid compounds were identified on the basis of comparing their retention times, UV-Vis spectra and mass spectrum data with the corresponding authentic standards. Concentrations were determined using an external calibration curve with citric acid (rT = 7.7 min; [M-H]⁺ 191 m/z), malic acid (rT = 9.1 min; [M-H]⁺ 133 m/z) and succinic acid (rT = 11.2 min; [M-H]⁺ 117 m/z).

2.2.5. Determination of total carotenoid content and HPLC analysis of individual carotenoids

The total carotenoid content determination was conducted according to Koca *et al.* [14], where 50 mg of dried sample were homogenised with 2 mL of hexane:acetone mixture (7:3). Carotenoid content was determined spectrophotometrically at 450 nm from the standard curve and expressed as micrograms of β -carotene equivalents (β -CE) per 100 mg of dw.

Individual carotenoids were determined from 50 mg of ground tissue and extracted in 1 mL cold ethanol and 0.8 mL hexane. The extraction mixture was centrifuged at 10,000 rpm for 5 min at 4 °C. The upper organic phase was transferred to a new microcentrifuge tube. In order to avoid losses, saturated sodium chloride (0.5 mL) and hexane (0.8 mL) were added twice to the pellet and the mixture was recentrifuged. The organic phase was free of visible particulates and was, therefore, not filtered. The organic phase was evaporated under a nitrogen stream. For the saponification of persimmon carotenoids, residues were dissolved in 1 mL of methanolic potassium hydroxide (2 M) and 0.1 % butylated hydroxytoluene was used to avoid oxidation. The mixture was left 3 h in the dark. It was transferred to a separatory funnel. Then 4 mL of 10 % sodium chloride, plus 2 mL of hexane, were poured over to remove potassium hydroxide. The hexane layer was also washed with bi-distilled water until the rinse had a neutral pH. Once again 2 mL of hexane were added to the potassium hydroxide to collect any remaining carotenoids. The combined hexane extracts were evaporated under a nitrogen stream. Samples were dissolved in 300 μ L of ethyl acetate and 10 μ L of were injected in reverse phase HPLC (Agilent 1200 series HPLC system (Santa Clara, CA)), including a model G1311A quaternary pump, a model G1367B autosampler, a model G1316A column oven and a model G13150 photodiode array detector. The column was an Agilent ZORBAX Eclipse XDB-C18, 5 μ m bead size, 4.6 mm x 150 mm, connected with an Eclipse XDB-C18 guard column. Column temperature was controlled at 30 °C during HPLC runs. Data were processed by the Agilent ChemStation software. Separation of carotenoids pigments was achieved by modifying a previously established gradient elution programme [15]. The flow rate was 1 mL min⁻¹. The mobile phases were acetonitrile: H₂O: triethylamine (900:99:1, v/v/v) (A) and ethyl acetate (B). The gradient elution programme was: 0-5 min, 100-75 % A; 5-10 min, 75-30 % A; 10-13 min, 30-0 % A; 13-14 min, 0-100 %; 14-15 min, 100 % A. Data were collected at 440 nm, 477 nm and 296 nm. Several carotenoids were identified based on retention time and spectrum

compared to commercially available authentic standards. Their quantity was extrapolated from standard curves of authentic compounds and was corrected for extraction efficiency based on the β -apo-8'-carotenal internal recovery standard. Concentrations were expressed as $\mu\text{g kg}^{-1}$ dw.

The vitamin A value in fruits was calculated as retinol activity equivalents (RAE) using the following conversion according to [16]: $\text{RAE} = [(\beta\text{-cryptoxanthin}/12) + (\alpha\text{-carotene}/12) + (\beta\text{-carotene}/6)]$.

2.3. Chemicals

The following standards were used to determine sugars, organic acids, soluble polyphenol content, carotenoids and antioxidant capacity: sucrose, fructose and glucose; citric acid, malic acid, succinic acid and fumaric acid; gallic acid; α/β -carotene, lutein, β -cryptoxanthin, violoxanthin, zeaxanthin; trolox solution from Sigma-Aldrich Chemie (Steinheim, Germany).

2.4. Statistical analysis

Data were subjected to an analysis of variance, and the multiple comparisons between means were determined by the least significant difference (LSD) test ($P \leq 0.05$) using the Statgraphics Plus 5.1 software application (Manugistics Inc., Rockville, MD, USA).

3. Results and discussion

3.1. External skin colour, flesh firmness, acetaldehyde production and sensory evaluation

External colour is the most widely used parameter as a non-destructive index for harvesting persimmon. In the present research, the fruits from all the cultivars were harvested in the commercial maturity stage, with an external colour index that ranged from 15 to 20, and corresponded to homogeneous skin coloration from full orange to reddish-orange, depending on the cultivar (data not shown). Firmness at harvest was above 30 N for all the studied cultivars, values which allow persimmons with a crisp texture to be marketed. The application of deastringency treatment with CO_2 to cultivars that were astringent at harvest neither modified fruit firmness nor skin colour. However, the

treatment brought about a sharp rise in acetaldehyde content from values below $0.5 \text{ mg } 100 \text{ mL}^{-1}$ at harvest to values between 1 and $3 \text{ mg } 100 \text{ mL}^{-1}$ (data not shown). This increase was due to exposing fruits to a high- CO_2 atmosphere since acetaldehyde accumulates under partially or totally anaerobic conditions [17].

The sensory evaluation corroborated absence of astringency at harvest in the fruits of non-astringent cultivars (data not shown). It also revealed that deastringency treatment was effective for removing astringency in all the astringent cultivars, except 'Hachiya', in which panellists detected slight astringency after applying treatment.

3.2. Study of soluble polyphenol content and antioxidant capacity

In persimmon, the soluble polyphenols that belong to the proanthocyanidin class are responsible for typical astringency since they interact with salivary proteins and give rise to the astringency sensation [18]. It is known that persimmons from both astringent and non-astringent cultivars are very astringent, with high soluble polyphenol content when fruit are small and immature. Nevertheless, while the fruits from astringent cultivars remain strongly astringent even when fruits are fully coloured, those from non-astringent cultivars lose astringency while growing on trees. It has been claimed that tannin cell development is continuous to late fruit growth stages in astringent cultivars, while tannin cells development stops in early fruit growth stages in non-astringent ones. Thus natural loss of astringency on trees is thought to be due to the dilution of tannins concentration on flesh as fruits grow [1].

In the present study, the fruits of the cultivars that were astringent at harvest showed high soluble polyphenol content, which ranged from $650 \text{ mg } 100 \text{ g}^{-1}$ in 'Rojo Brillante' to $1,220 \text{ mg } 100 \text{ g}^{-1}$ in 'Giombo' (Figure 1A). Major differences in the soluble polyphenol contents among cultivars have been previously reported by Veberic *et al.* [8]. In contrast, the soluble polyphenol content of non-astringent cultivars was much lower, and they all showed values below $185 \text{ mg } 100 \text{ g}^{-1}$.

As expected, when persimmons from astringent cultivars were subjected to deastringency treatment with a high CO₂ concentration, soluble polyphenols drastically dropped, and similar values to those of non-astringent cultivars were given. The decline in soluble polyphenols in astringent cultivars ran in parallel to the sharp rise in acetaldehyde content observed after deastringency treatment. It is known that the insolubilisation of tannins, and consequently astringency removal, are mediated by the acetaldehyde generated under anaerobic conditions [13,19].

After deastringency treatment, the concentration of soluble polyphenols vastly varied among cultivars; the highest values, 200 mg 100 g⁻¹, were observed in cultivar 'Hachiya' which agrees with the sensory astringency detected by the panellists. Currently, there is no established threshold of soluble tannins that ensures lack of astringency in persimmon fruits. In fact, perception of astringency has been shown to be influenced by presence of other compounds, like acids and sugars [20,21] and it is probably affected by the specific tannin composition of each cultivar [22]. However, it must be noted that after applying deastringency treatment to astringent cultivars, the concentration of soluble tannins lowered to similar values to those of naturally non-astringent cultivars.

The higher antioxidant capacity exhibited by astringency cultivars at harvest compared to non-astringent ones (Figure 1B) was related directly to content of polyphenols, which possess strong radical scavenging activity. It has been reported that polyphenols are the major antioxidant compounds in persimmon pulp [23]. Non-astringent cultivars gave values below 6 µmol TE 100 mg⁻¹. However, the content among astringent cultivars varied vastly and presented values that ranged from 25 to 60 µmol TE 100 mg⁻¹ (Figure 1B). Similar results were obtained by Katsube *et al.* [24], who measured antioxidant activity by the DPPH radical method, and obtained more than one order magnitude higher in astringent persimmons than in non-astringent ones.

As observed with soluble polyphenol content, total antioxidant capacity also significantly lowered after subjecting astringent cultivars to deastringency treatment. Thus after CO₂ treatment, most cultivars showed similar total antioxidant capacity values to those observed by non-astringent cultivars. The highest values, 10 µmol TE 100 mg⁻¹, were detected in cultivar 'Hachiya' while the lowest values, below 2 µmol TE 100 mg⁻¹, were given by 'Rojo Brillante', 'Tone Wase' and 'Kaki Tipo' (Figure 1B). These results agree with those of Del Bubba *et al.* [9], who reported that the deastringency treatment with CO₂

applied to cultivars ‘Kaki Tipo’ and ‘Rojo Brillante’ led to a 10-fold drop in antioxidant capacity.

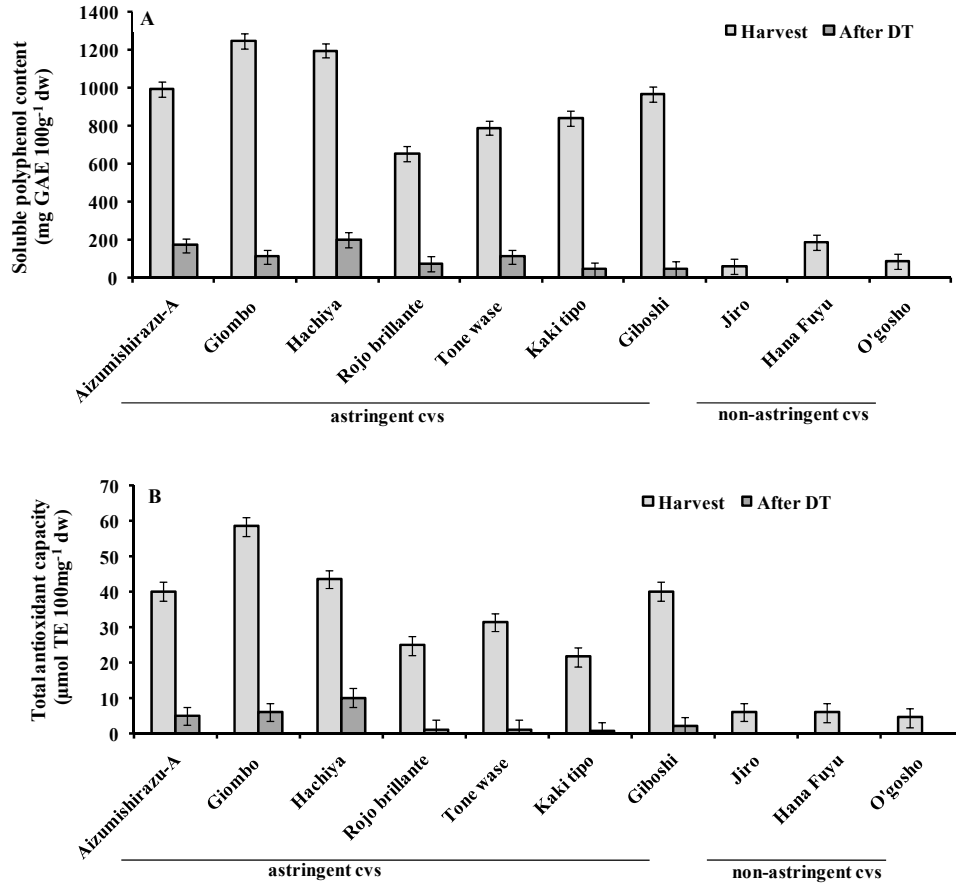


Fig. 1. Soluble polyphenol content (A) and total antioxidant capacity (B) of ten persimmon cultivars at harvest and after destringency treatment (DT). Vertical bars denote the LSD interval ($P < 0.05$).

3.3. Study of sugars

Table 1 shows the individual and total sugars in the 10 studied persimmon cultivars. The main sugars found in the flesh of all persimmon cultivars were sucrose, glucose and fructose, which agrees with previously reported studies [8,9,25].

After taking into account the individual and total sugars of astringent cultivars at harvest, 'Tone wase' and 'kaki Tipo' presented the highest content of glucose (509.9-394.4 $\mu\text{g mg}^{-1}$) and fructose (412.1- 335.7 $\mu\text{g mg}^{-1}$) and total sugars (1086-996.7 $\mu\text{g mg}^{-1}$). The highest sucrose content value went to 'Giombo' (401.8 $\mu\text{g mg}^{-1}$), 'Rojo Brillante' (396.9 $\mu\text{g mg}^{-1}$) and 'Kaki Tipo' (356.5 $\mu\text{g mg}^{-1}$), while 'Tone Wase' obtained the lowest value (74.7 $\mu\text{g mg}^{-1}$). Cultivars 'Aizumishirazu-A', 'Hachiya' and 'Giboshi' showed the lowest sugar content values, similarly to that observed in non-astringent cultivars. The total sugar content in non-astringent cultivars was lower, and ranged from 433.6 to 609.4 $\mu\text{g mg}^{-1}$. Cultivar 'Hana Fuyu' presented a higher content of reducing sugars (glucose and fructose) than sucrose, while sucrose was found at the highest concentration, followed by glucose and fructose in 'Jiro' and 'O'goshō'.

The effect of deastringency treatment on total sugar content strongly depends on the cultivar. While the total sugar content of 'Aizumishirazu-A', 'Hachiya' and 'Giboshi' was not affected by deastringency treatment, in 'Tone Wase', 'Rojo Brillante', 'Kaki Tipo' and 'Giombo', the cultivars that presented the highest sugars level at harvest, deastringency treatment caused an important drop in the total sugar content to about half the original value. So when the fruits of all the astringent cultivars were ready to be marketed (after treatment deastringency) exhibited similar total sugars content to that of non-astringent cultivars.

Reduced total sugars after deastringency treatment was generally due to the sharp drop in sucrose in most cultivars, which even became undetectable in cultivars such as 'Hachiya', 'Tone Wase', 'Kaki Tipo' and 'Giboshi'. It is noteworthy that cultivar 'Aizumishirazu-A' was the only cultivar that showed no differences in sucrose content between values at harvest and those recorded after applying CO₂ treatment. Glucose and fructose were much less affected by deastringency treatment. Glucose and fructose contents remained almost constant in most cultivars, except cultivars 'Rojo Brillante' and 'Tone Wase'. Glucose in 'Rojo Brillante' significantly decreased after applying treatment (from values of 317.7 $\mu\text{g mg}^{-1}$ at harvest to values of 158.8 $\mu\text{g mg}^{-1}$ after

treatment), while 'Tone Wase' showed a more reduced decline for glucose (from values of 509.9 $\mu\text{g mg}^{-1}$ to 323.5 $\mu\text{g mg}^{-1}$) and fructose (from 412.1 $\mu\text{g mg}^{-1}$ to 301.5 $\mu\text{g mg}^{-1}$). The results obtained in the present work agree with Ittah [25], who reported that deastringency treatment in CO_2 atmospheres reduces the total sugars content and diminishes sucrose starts when CO_2 treatment commences, while changes in glucose and fructose content are very gradual. This reduction in total sugars due with deastringency treatment can be explained by a possible glycoside formation between soluble tannins and soluble sugars.

The reduced sucrose content in most cultivars after deastringency treatment could be due to induced invertase activity through the tannin insolubilisation process during deastringency. In previous studies, the decline of sucrose that occurred in some astringent persimmon fruits during fruit growth and ripening have been attributed to an increase of invertase activity coupled with a decrease of soluble tannins [9] as an *in vitro* study has demonstrated the strong inhibition of this enzyme by gallic and tannin acids [26]. It has also been reported that fruit enzymatic systems, such as sucrose synthetase, invertase, fructose isomerase, cellulose and enzymes from the gluconeogenesis pathway, are involved with fruits under anaerobic conditions [27,28].

Table 1. Sugars content ($\mu\text{g mg}^{-1} \text{dw}^{\text{a}}$) of ten persimmon cultivars at harvest and after destringency treatment (DT).

	Sucrose		Glucose		Fructose		Total sugars	
	Harvest	After DT	Harvest	After DT	Harvest	After DT	Harvest	After DT
<i>astrigent cvs</i>								
Azumishirazu-A	241.1 \pm 6.3 de	248.9 \pm 10.2 de	194.5 \pm 6.1 bc	200.4 \pm 7.8 bc	171.9 \pm 5.9 bc	177.1 \pm 5.6 bc	607.5 \pm 18 abc	626.5 \pm 23.3 bc
Giombo	401.8 \pm 28.4 f	190.2 \pm 34.7 bc	240.1 \pm 18.9 bcd	196.6 \pm 6.3 bc	201.7 \pm 15.6 bcd	178.1 \pm 3.5 bc	843.8 \pm 55 de	564.9 \pm 37.5 ab
Hachiya	147.6 \pm 25.8 ab	nd	281.5 \pm 44.5 def	337.5 \pm 9.9 gh	232.6 \pm 24.2 cde	288.7 \pm 17.8 fgh	661.7 \pm 45.3 bc	626.2 \pm 27.1 bc
Rojo brillante	396.9 \pm 13.4 f	222.0 \pm 10.9 bcd	317.7 \pm 13.6 fg	158.8 \pm 12.4 ab	263.9 \pm 10 efg	265.9 \pm 15.1 efg	978.6 \pm 13.3 ef	646.8 \pm 14 bc
Tone wase	74.7 \pm 11.4 a	nd	509.9 \pm 1.4 i	323.5 \pm 12.8 fg	412.1 \pm 9 i	301.5 \pm 11.7 fgh	996.7 \pm 19.5 ef	625.1 \pm 22.7 bc
Kaki tipo	356.5 \pm 51.7 f	nd	394.4 \pm 36.1 h	348.5 \pm 31.7 gh	335.7 \pm 28.2 h	318.8 \pm 34.4 gh	1086.6 \pm 114.7 f	667.3 \pm 81.2 bc
Giboshi	143.3 \pm 25.4 ab	nd	204.3 \pm 39.5 bc	265.4 \pm 18.5 cde	183.7 \pm 17.8 bc	250.8 \pm 15.1 def	531.3 \pm 81.8 ab	516.2 \pm 33.5 ab
<i>non-astriгент cvs</i>								
Jiro	310.8 \pm 55.8 ef	-	152.16 \pm 2.8 ab	-	146.5 \pm 4.9 ab	-	516.2 \pm 57.1 ab	-
Hana Fuyu	199.0 \pm 4.6 bc	-	295.9 \pm 33.3 def	-	277.2 \pm 34.8 fgh	-	609.4 \pm 64.7 bc	-
O'goshio	224.8 \pm 31.2 bcd	-	110.8 \pm 14 a	-	97.9 \pm 18 a	-	433.6 \pm 72.7 a	-

^a Values are the mean of three independent determinations. For each sugar, values with different letters indicate significant differences ($P < 0.05$). *nd*: not detected.

3.4. Study of organic acids

Citric acid, malic acid and succinic acid were the main organic acids found in the present study, while fumaric acid was found in trace amounts (Table 2). At harvest, the total organic acids content ranged from $15.8 \mu\text{g mg}^{-1}$ to $30.9 \mu\text{g mg}^{-1}$. Citric acid content was over $5.3 \mu\text{g mg}^{-1}$ among all the cultivars, except non-astringent cultivars 'Hana Fuyu' and 'O'goshō', which presented lower values. Malic acid content was over $10 \mu\text{g mg}^{-1}$ in astringent cultivars such as 'Rojo brillante', 'Tone wase', 'Kaki tipo', and also in non-astringent cultivars 'Jiro' and 'O'goshō'. The lowest value of this acid was shown for 'Giombo' ($4.3 \mu\text{g mg}^{-1}$). It is noteworthy that succinic acid content was higher in astringent cultivars, and 'Giombo' is highlighted because it showed the highest values ($19.3 \mu\text{g mg}^{-1}$). Non-astringent cultivars gave values below $5 \mu\text{g mg}^{-1}$ (Table2).

Although the effect of deastringency treatment on organic acids has not been previously addressed, a reduction in their content was expected since it is known that high CO_2 atmospheres induce changes in organic acid metabolism in other fruits, such as strawberry or cherimoya [29,30]. Nevertheless in the present study, deastringency treatment did not affect the total organics acid content, and slightly modified individual organic acids.

The organic acids content found in the present study was higher than those reported by Veberic *et al.* [8], which is not surprising given the maturity stage of the fruit evaluated in each case. Fruits were analysed herein in the 'ready-to-eat' maturity stage, while the evaluations of Veberic *et al.* [8] were made with fully ripened fruit. It has been reported that during fruit ripening, metabolic processes led to a considerable loss in organic acid in persimmon fruits [31].

Table 2. Organic acids content ($\mu\text{g mg}^{-1} \text{dw}$)^a of ten persimmon cultivars at harvest and after destringency treatment (DT).

	Citric acid		Malic acid		Succinic acid		Total organic acids	
	Harvest	After DT	Harvest	After DT	Harvest	After DT	Harvest	After DT
<i>astrigent cvs</i>								
Azumishirazu-A	6.2 ± 0.5 bc	5.3 ± 0.3 b	6.2 ± 0.3 abc	8.3 ± 0.4 c	6.1 ± 0.4 ab	5.9 ± 0.2 ab	18.5 ± 1.0 ab	19.5 ± 0.7 bc
Giombo	7.3 ± 0.3 d	6 ± 0.2 bc	4.3 ± 0.4 a	5.2 ± 0.3 ab	19.3 ± 1.3 g	16.6 ± 0.6 f	30.9 ± 1.3 f	27.8 ± 0.6 ef
Hachiya	6.4 ± 0.3 bcd	5.6 ± 0.4 b	6.4 ± 0.1 abc	7.6 ± 0.1 bc	8.7 ± 0.4 cd	8.6 ± 0.8 cd	21.5 ± 0.7 bc	21.8 ± 1.1 bc
Rojito brillante	5.3 ± 0.2 b	5.7 ± 0.3 b	11.1 ± 0.5 de	8.7 ± 0.9 cd	8 ± 0.2 cd	9.2 ± 0.3 d	24.4 ± 0.7 d	23.6 ± 1.1 d
Tone wase	6.7 ± 0.3 bcd	7 ± 0.5 d	11.1 ± 2.1 e	12.3 ± 0.8 e	8 ± 0.2 cd	7.5 ± 0.6 bcd	25.8 ± 2.2 de	26.8 ± 1.5 de
Kaki tipo	6.8 ± 0.4 bcd	5.5 ± 0.2 b	10.7 ± 0.4 de	10.8 ± 1.6 de	6.2 ± 0.5 ab	7.1 ± 0.1 bc	23.7 ± 0.6 d	23.4 ± 1.4 d
Giboshi	7.2 ± 0.4 d	6.4 ± 0.3 bcd	7.2 ± 0.2 bc	8.4 ± 1.0 c	11.7 ± 1.3 e	12.2 ± 0.7 e	26.1 ± 1.8 de	27 ± 1.4 de
<i>non-astriгент cvs</i>								
Jiro	6 ± 0.1 bc	-	11.3 ± 0.6 e	-	4.8 ± 0.1 a	-	22.1 ± 0.6 cd	-
Hana Fuyu	4.7 ± 0.4 ab	-	6.6 ± 0.8 abc	-	4.5 ± 0.1 a	-	15.8 ± 0.8 a	-
O'goshi	3.8 ± 0.2 a	-	11.5 ± 0.7 e	-	4.5 ± 0.2 a	-	19.8 ± 0.8 bc	-

^a Values are the mean of three independent determinations. For each organic acid, values with different letters indicate significant differences ($P < 0.05$).

3.5. Study of total and individual carotenoids

Figure 2 depicts the total carotenoid content at harvest and after submitting astringent cultivars to the CO₂ treatment. At harvest the total content greatly varied among the ten cultivars; cultivar ‘Hachiya’ had the highest content (above 100 µg β-CE 100 g⁻¹), while the lowest values went to cultivar ‘Rojo Brillante’ and ‘O’goshō’, with 20 and 23.5 µg β-CE 100 g⁻¹, respectively. All the other cultivars showed values that ranged from 40 to 80 µg β-CE 100 g⁻¹.

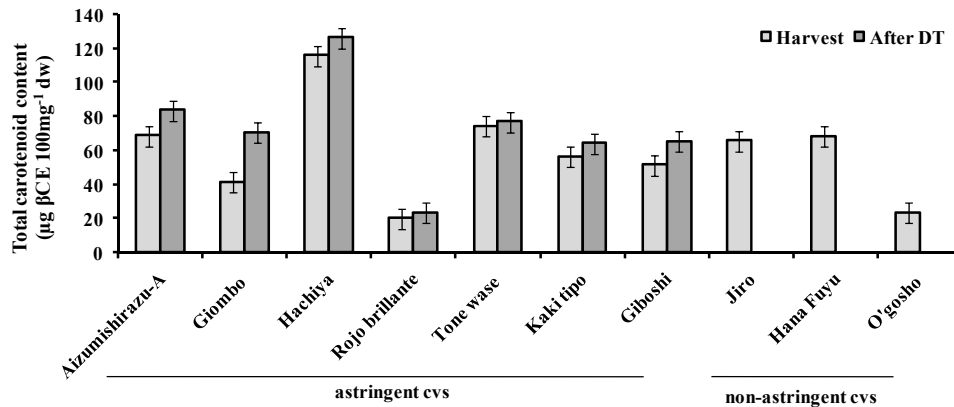


Fig. 2. Total carotenoid content of ten persimmon cultivars at harvest and after deastringency treatment (DT). Vertical bars denote the LSD interval ($P < 0.05$).

The individual carotenoids detected by HPLC in persimmon flesh were lutein, violoxanthin, zeaxanthin, β-cryptoxanthin and β-carotene (Table 3). β-cryptoxanthin was the predominant carotenoid found at harvest in all the studied cultivars. This xanthophyll has also been reported in previous studies as the main carotenoid in persimmon flesh [8,10,32-34]. The relative levels among zeaxanthin, lutein and β-carotene were cultivar-dependent. Violoxanthin was the lowest carotenoid found in all the cultivars.

Table 3. Carotenoids content ($\mu\text{g}/\text{kg dw}$)^a of ten persimmon cultivars at harvest and after deastringency treatment (DT).

	Lutein		Vioioxanthin		Zeaxanthin		β -Cryptoxanthin		β -Carotene		RAE	
	Harvest	After DT	Harvest	After DT	Harvest	After DT	Harvest	After DT	Harvest	After DT	Harvest	After DT
<i>astrigent cvs</i>												
Aizumishirazu-A	183.9 ± 17bc	422.6 ± 28.1gh	22.2 ± 5.1a	60.4 ± 0.4cd	231.6 ± 14.9de	298.1 ± 4.5fg	392.3 ± 33.5bcd	547.4 ± 31.8def	89.9 ± 5.9bcd	158 ± 21.9g	47.7 ± 2.6cd	71.9 ± 6fg
Gombo	69.6 ± 12.5a	328.2 ± 8.1ef	45.6 ± 10.abcd	112.7 ± 5.1ef	134.9 ± 2.1bc	212.2 ± 5.2de	267.9 ± 11.5b	706.6 ± 23.4e	68.9 ± 5.8ab	112.7 ± 1.6def	33.8 ± 1.9b	77.7 ± 2.2gh
Hachiya	235.2 ± 41.5cd	229.8 ± 9.8cd	21.2 ± 0.9a	25.4 ± 3.2ab	445.4 ± 30.6h	397.3 ± 25.1h	771.1 ± 71.3e	801.8 ± 28.7e	213.1 ± 22.3h	212 ± 7h	99.8 ± 7.6i	102.2 ± 1.2i
Rojo brillante	97.8 ± 12.1ab	225.4 ± 53.1cd	33.7 ± 1.9abc	54.8 ± 12.5bcd	70.1 ± 5.8a	84.6 ± 17.3ab	325.4 ± 34.1b	291.3 ± 55.7b	70.4 ± 1.7ab	90.1 ± 14.8bcd	38.9 ± 3.1bc	39.3 ± 0.2bc
Tone wase	445.7 ± 30.1h	462.7 ± 19.iii	49.1 ± 15.3abcd	64.9 ± 2.7d	261.5 ± 37.ef	334.5 ± 8.5g	510.4 ± 49.0cd	459.1 ± 23.3cd	80.7 ± 3.7abc	95.6 ± 6.1bcde	56 ± 6.8de	54.2 ± 1.5def
Kaki tipo	545.59 ± 15.1	429.8 ± 56.4gh	95.9 ± 6.3e	99.8 ± 16e	224.2 ± 9.5de	180.6 ± 13.6cd	508.7 ± 24.0cd	538.9 ± 27.2d	106.1 ± 4.3cde	119 ± 7.9def	60.1 ± 0.9def	64.7 ± 3.1ef
Gboshi	350.2 ± 8.8efg	382.1 ± 26.6efgh	132.8 ± 2.6fg	154.1 ± 18.4g	259.7 ± 20.0ef	263.7 ± 17.1ef	488.9 ± 32.5cd	686.1 ± 65.5e	102.7 ± 11.2cde	143.6 ± 9.7fg	57.9 ± 3.2de	81.1 ± 6gh
<i>non-astriгент cvs</i>												
Jiro	292.7 ± 61.2de	-	60 ± 16.6cd	-	177.9 ± 31.0de	-	800.8 ± 23.1e	-	121.6 ± 4.2ef	-	87 ± 3.4h	-
Hana Fuyu	296.5 ± 31.3def	-	95.1 ± 15.3e	-	292.2 ± 18.9gh	-	502.9 ± 50.9cd	-	121 ± 16.1def	-	62.1 ± 6.8ef	-
Ogoshu	39.7 ± 12.5a	-	27.1 ± 8.4ab	-	100.4 ± 11.6ab	-	90.8 ± 9.2a	-	56.3 ± 7.9a	-	17 ± 2.1a	-

^a Values are the mean of three independent determinations. For each carotenoid, values with different letters indicate significant differences ($P < 0.05$).

Deastringency treatment led to an increase in the total carotenoids content compared to the values recorded at harvest in some cultivars, such as 'Aizumishirazu-A', 'Giombo' and 'Giboshi' (Figure 2), which coincides with the changes observed in individual carotenoids (Table 3). Thus the content of all the individual carotenoids content in cultivars 'Aizumishirazu-A' and 'Giombo' significantly increased after CO₂ treatment, while the content of β -cryptoxanthin and β -carotene in 'Giboshi' mainly increased. Very few studies have addressed the effect of deastringency treatment on carotenoid content in persimmon. Plaza *et al.* [35] observed that the carotenoids content of persimmons subjected to high pressure remained unchanged when applied to fruits in a mid-season maturity stage. Yet when this treatment was assayed in fruits in an advanced maturity stage, it led to increased carotenoids content, probably due to modifications in their bioaccessibility. The retinal activity equivalent (RAE) provided information about dietary intake of provitamin A carotenoids. In the present study, cultivars 'Jiro' and 'Hachiya' presented the highest RAE values (102.14 and 126.99 $\mu\text{g kg}^{-1}$, respectively), mainly due to their high β -cryptoxanthin content.

4. Conclusions

Persimmons harvested with a firm texture that are to be commercialised as "ready-to-eat crisp" are rich in sugars (glucose, fructose and sucrose), organic acids (citric acid, malic acid and succinic) and carotenoids (β -cryptoxanthin, lutein, violoxanthin, zeaxanthin, and β -carotene). The relative content of these compounds heavily depends on the cultivar, but astringent cultivars generally present a higher content of sugars and organic acids than non-astringent ones. At harvest, the fruits of astringent cultivars show especially high soluble polyphenols content and total antioxidant capacity. However when this fruit was submitted to deastringency treatment with CO₂, the values of both soluble polyphenols content and total antioxidant capacity lowered, similarly to those of the naturally non-astringent fruits. Deastringency treatment also induced changes in fruit carotenoids and sugar composition.

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IV. GENERAL DISCUSSION

IV.1. INVOLVEMENT OF OXIDATIVE STRESS IN PHYSIOLOGICAL DISORDERS OF PERSIMMON FRUITS

IV.1.1. Deastringency treatment and mechanical damage disrupt the fruit redox state by inducing flesh browning

Flesh browning is one of the main postharvest disorders in persimmon fruit cv. Rojo Brillante, which leads to significant postharvest loss. Previous studies have claimed that the mechanical damage suffered by fruits during packing operations may be a decisive factor of browning manifestation (Besada *et al.*, 2010a). Astringency level has also been found to influence sensitivity to browning, which suggests that tannins are involved in this disorder (Besada *et al.*, 2012). In other fruits, browning manifestation is also associated with alteration of phenolic compounds (Lee *et al.*, 2005). However, the biochemical mechanism behind this disorder in persimmons was unclear.

In this Thesis, microstructural and biochemical studies were conducted to elucidate the mechanism behind this disorder.

The study performed with fruits submitted to different CO₂ exposures (from 0 h to 40 h) showed that only the fruits submitted to mechanical damage manifested flesh disorders. However, damage manifestation very much depended on astringency level. So flesh browning was observed only in fruits previously submitted to CO₂, while a new disorder, described and named “pinkish bruising”, was associated for the first time with fruits with a high astringency level. Therefore, mechanical impacts were herein confirmed as the factor that triggers not only browning, but also pinkish-bruising development. The incidence of the two flesh disorders also correlated well with the level of soluble tannins present in fruits at the time of mechanical damage. Pinkish-bruising correlated positively with soluble tannins content, while browning correlated inversely.

Both the browning and pinkish-bruising disorders were related to microstructural changes by Optical and Cryo-SEM studies; pinkish-bruising was found to be an output of cellular content and the appearance of pink insoluble material in intercellular spaces, which apparently results from an insolubilisation process of the initial soluble material after leaving cells. Flesh browning was associated with presence of large-sized cells of an intense red-

brown colour, filled with an insoluble material, identified as tannic cells, in which tannins insolubilisation took place during CO₂ treatment.

The chromatographic study revealed that the changes in insoluble tannins associated with browning manifestation showed the same pattern as those changes associated with controlled tannins oxidation by the addition of ROS generator KO₂, which suggests that the tannins oxidation process is behind flesh browning manifestation. It must be noted that the fruits overexposed to CO₂ (36-40 h) exhibited more intense browning than the 24 hour-treated fruit, although the soluble tannins level was similar. This fact suggests that not only tannins, but also CO₂ treatment itself, must be involved in disorder development.

Accordingly, studying the redox system by means of *in vivo* staining revealed that CO₂ treatment substantially altered redox state of the fruit. After studying reactive oxygen species (ROS) in the fruits subjected to destringency treatment, significant O₂⁻ accumulation took place with hours of exposure to CO₂ treatment. This fact was associated with accumulation of acetaldehyde as it represents a possible source of hypoxia-stimulated ROS production. Xanthine oxidase (XOD) is a key enzyme responsible for initial dioxygen activation, and this enzyme can use acetaldehyde as electron donors (Bolwell & Wojtaszek, 1997). In other studies, anaerobic atmospheres have also been described as one of the environmental stresses that induces oxidative burst in plants (Blokina *et al.*, 2003).

A biochemical and enzymatic study was conducted to study the effect of destringency treatment on the redox system in-depth. It revealed that activity of ROS scavenging enzymes (SOD, CAT and APX), as a response to the increased ROS levels caused by treatment, was enhanced. The total antioxidant capacity of the fruit drastically decreased in association with tannins insolubilisation due to CO₂ treatment. It is well-known that soluble tannins of persimmon pulp possess strong radical scavenging activity (Gu *et al.*, 2008).

We must bear in mind that although CO₂ treatment leads to an oxidative stress state, the treatment itself does not result in flesh disorders, and mechanical damage is necessary to trigger both browning and pinkish-bruising disorders in persimmon. This fact was clarified by the biochemical study with the astringent and non-astringent fruits submitted to mechanical impacts. This study revealed that mechanical impacts also triggered oxidative stress, and that

both O_2^- and H_2O_2 , accumulated considerably in both astringent and non-astringent fruits.

The imbalance in the redox state, associated with both CO_2 deastringency treatment and mechanical damage, could lead to a tannins oxidation process, which would result in the manifestation of flesh disorders according to the soluble/insoluble tannins level. Thus flesh browning was manifested in the fruits previously subjected to deastringency treatment, while pinkish-bruising was manifested only in the fruits that remained astringent.

By taking into account the implication of tannins in the flesh disorders of 'Rojo Brillante' persimmon, the influence of persimmon type (astringent and non-astringent cultivars) on flesh disorders sensitivity was studied. Our results strongly suggested that fruit sensitivity to the flesh disorders associated with mechanical damage is clearly influenced by persimmon type. So similarly to 'Rojo Brillante', all the tested astringent cultivars displayed browning and/or pinkish-bruising in mechanical-packaged fruits according to the insoluble/soluble tannins level. Nevertheless, non-astringent cultivars did not exhibit the browning/pinkish-bruising associated with mechanical damage, even when fruits had been previously submitted to CO_2 treatment; in these cultivars, mechanical damage resulted in uncoloured bruised flesh areas. Although more studies are required to understand the influence of persimmon type on the susceptibility of fruits to manifest the flesh disorder induced by mechanical damage, it is quite likely that the effect of the CO_2 treatment on the redox system vastly differs between astringent and non-astringent cultivars. Indeed in astringent cultivars, CO_2 treatment lead to a drastic decrease of total antioxidant capacity in parallel to the tannins insolubilisation process, while in non-astringent cultivars no changes in antioxidant capacity are associated with CO_2 treatment since tannins are insoluble at harvest.

IV.1.2. The redox system is involved in chilling injury and its alleviation by 1-methylcyclopropene

Chilling injury (CI) is another physiological disorder that occurs in many plants and fruits, particularly those of a tropical and subtropical origin, like persimmon, as a result of their exposure to low, but non-freezing, temperatures (Jackman *et al.*, 1988). The symptoms of this disorder in persimmon vary

among cultivars, but are always generally associated with major changes in flesh texture.

In 'Rojo Brillante', the effect of cold storage on the development of this disorder has been widely studied. The main CI symptom is a drastic flesh softening, mainly manifested when fruit is transferred from low to shelf-life temperatures. This symptom is the result of the degradation of the cell wall material with loss of intercellular adhesion (Pérez-Munuera *et al.*, 2009) as reported in other cultivars (Grant *et al.*, 1992).

As with many other abiotic stresses, extreme temperatures are known to affect the redox state of fruits and plants. In this Thesis the study performed in 'Rojo Brillante' revealed that low-temperature exposure led to an increase in both H₂O₂ content and the antioxidant activity of enzymes, such as APX, CAT, SOD and LOX, which were enhanced with exposure time to cold storage. After transferring fruits from low to shelf-life temperatures, an oxidative burst, with major H₂O₂ accumulation and a sharp increase in CAT, POD and LOX activity, was exhibited in parallel to the softening CI manifestation. This oxidative stress was more marked the longer the previous cold storage period lasted. In 'Fuyu' persimmon, CI manifestation as flesh gelling has also been associated with changes in the fruit redox system (Zhang *et al.*, 2010).

The use of treatments to control chilling injury becomes necessary to store persimmon at low temperature. In this way, 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, has been shown to reduce CI symptoms in a large number of persimmon cultivars (Girardi *et al.*, 2003; Salvador *et al.*, 2004a; Tibola *et al.*, 2005). Specifically in 'Rojo Brillante' persimmon, 1-MCP reduces low temperature-induced flesh softening by preserving the integrity of cell walls and adhesion between adjacent cells (Salvador *et al.*, 2004a; 2004b; Pérez-Munuera *et al.*, 2009).

In the present Thesis the implication of redox system on the 1-MCP chilling injury alleviation was evaluated. Our results indicated no differences in either H₂O₂ content or in the activity of pro- and antioxidant enzymes between untreated and 1-MCP-treated fruits while fruits were exposed to low temperature. Therefore, 1-MCP treatment did not reduce the progressive oxidative stress associated with low-temperature exposure.

However, during shelf-life the changes in the redox system were highly affected by the 1-MCP treatment. So, the 1-MCP-treated fruit showed down-regulated POD activity and up-regulated CAT activity, which resulted in slower H₂O₂ accumulation. Reduction in flesh softening, as the main CI manifestation in 'Rojo Brillante' persimmon by 1-MCP, was associated with the modulation of the redox state of fruits during the shelf-life period following low-temperature storage. The obtained results allowed us to understand why the CI reduction by 1-MCP treatment in 'Rojo Brillante' persimmon, which occurs during shelf-life, is similar when 1-MCP was applied before cold storage or immediately before transferring fruits to shelf-life conditions (Salvador *et al.*, 2004a). Similarly in 'Fuyu' persimmon, the effect of 1-MCP on CI reduction was associated with the mitigation of oxidative stress during the symptoms manifestation period (Zhang *et al.*, 2010).

IV.2. EVALUATION OF POSTHARVEST TREATMENTS TO IMPROVE FRUIT QUALITY DURING COLD STORAGE

IV.2.1. Application of controlled atmospheres to alleviate the chilling injury of different cultivars

The use of controlled atmospheres (CA) is currently a frequently used technology to preserve the quality of pears, apples and other commodities (Kader, 2002) during cold storage. In persimmon fruits, the effect of CA on extending storage has been widely studied in non-astringent cultivar 'Fuyu' (Burmeister *et al.*, 1997; Donazzolo & Brackmann, 2002; Lee *et al.*, 2003; Park & Lee, 2008; Woolf & Ben-Arie, 2011; Brackmann *et al.*, 2013). However, the incidence of skin and flesh disorders is a limitation to store this cultivar in a CA. To date, studies into CA storage have been limited to other cultivars.

In this context, the effect of combining 1-MCP pre-treatment and a CA based on 4-5% O₂ + N₂ during the cold storage of cultivars 'Rojo Brillante' and 'Triumph' was studied herein. Our results showed that the response of both cultivars to this technology significantly differed. In 'Triumph', the combined use of 1-MCP and CA alleviated fruit softening and gelling, which allowed the storage of this cultivar to be prolonged to up to 3 months. Although this combined treatment in 'Rojo Brillante' also retarded fruit softening and

inhibited gelling development, it led to internal browning, which is a major limiting factor. Therefore, our results revealed that tolerance to a low-oxygen atmosphere vastly differs among cultivars, which corroborates the importance of investigating an adequate atmosphere for each one.

Irrespective of the cultivar, the evaluated 4-5% O₂ + N₂ atmosphere had an effect on retarding black spot caused by *Alternaria alternata*, which is a pathogen that usually colonises persimmon fruit during low-temperature storage (Krammes *et al.*, 2006; Palou *et al.*, 2009; Kobilier *et al.*, 2011). Accordingly, it has been previously reported that an atmosphere based on low O₂ levels may have a fungistatic effect by inhibiting spore germination and fungus development (Neuwald *et al.*, 2005).

Although the continuous application of a specific CA during storage is the most usual technology, short-term anoxic treatments applied before prolonged storage have also been reported to alleviate CI in fruits such as avocado, kiwi or peach (Pesis *et al.*, 1993; Polenta *et al.*, 2005; Liu *et al.*, 2007; Song *et al.*, 2009). In this sense, treatments based on a high CO₂ concentration (80-95%) for 12-24 h, which is usually applied to remove astringency from persimmon cultivars that are astringent at harvest, may be considered a short-term anoxic treatment. However, no effect of CI control has been observed.

By taking into account that alleviating CI by short-term anoxia has been associated with enhancing the activity of ROS scavenger enzymes in several fruits (Liu *et al.*, 2007; Song *et al.*, 2009; Gao *et al.*, 2009), and that CO₂ treatment modifies the redox system in astringent persimmon fruits, as mentioned above, we considered that studying the response of non-astringent persimmons to short-term high CO₂ treatments, which has been never approached to date, is interesting. Exposure to a high CO₂ concentration for 12-36 h of non-astringent cultivar 'Fuyu' had a positive effect on CI alleviation; the longer the CO₂ treatment, the greater the alleviation of CI symptoms such as flesh gelling and fruit darkening. Microstructural studies revealed that alleviation of CI, specifically the flesh gelling symptom, was based on CO₂ treatment preserving the integrity of cell walls during storage. However, this treatment did not avoid flesh softening during the shelf-life period.

In this Thesis, CI manifestation was related to gradual oxidative stress during cold storage and posterior shelf-life, and CO₂ treatment is also reported to alter the fruit redox system by showing increased ROS levels and the

consequent activation of ROS scavenging enzymes. Therefore, it is likely that short-term high CO₂ treatments act as mild stress exposure, which would enhance the cell's capacity to resist further or future exposure to oxidative stress (Toivonen, 2003).

It should be noted that this effect seemed strongly dependent on the cultivar since it has not been previously described in astringent cultivars in which CO₂ treatment is commonly applied. It is noteworthy that applying CO₂ treatment to astringent cultivars drastically diminished antioxidant capacity, in parallel to the tannins insolubilisation process, while tannins naturally took their insoluble form in non-astringent cultivars. This fact may be a determinant for the effect of CO₂ treatment on the fruit redox stage.

Therefore, while this technology can be considered a useful tool to control CI in 'Fuyu' persimmon, further studies are needed to know the response of other non-astringent cultivars.

IV.2.2. 1-Methylcyclopropene reduces fruit softening induced by ethyl formate disinfection treatment

In some countries (e.g., New Zealand, Australia, USA, South Korea, Japan), presence of quarantine pests, such as Queensland fruit fly (QFF) and mealy bugs, are major constraints to commercialise persimmons on export markets.

Current disinfestation treatments are heavily dependent on the use of fumigant methyl bromide (MeBr), which is an ozone-depleting substance whose use is under constant pressure. Therefore, new "soft" solutions, which leave no residues, have been studied in recent years. Along these lines, ethyl formate (EF) is a plant volatile compound that has been used as an alternative method to MeBr treatment for insect disinfestations in several horticultural products, such as table grapes, onions, dates and apricots (Simpson *et al.*, 2007; Van Epenhuijsen *et al.*, 2007; Finkelman *et al.*, 2010; Chhagan *et al.*, 2013). EF is considered a Generally Recognized as Safe (GRAS) product and can potentially degrade to biogenic levels in tissues of treated commodities before the product reaches the market. EF fumigation has proven effective for controlling mainly

surface insects: spider mite, western flower thrips, omnivorous leafroller, aphids, mealy bugs and black widow spiders (Simpson *et al.*, 2004; Simpson *et al.*, 2007; Finkelman *et al.*, 2010; Cho *et al.*, 2012). However fruit quality disorders, such as calyx damage in strawberries (Simpson *et al.*, 2004) or rachis browning in table grapes (Simpson *et al.*, 2007), have been associated with its use. In persimmons, recent studies have shown that EF treatment successfully disinfects fruit. Nonetheless, the main limiting factor for its use is that it promotes fruit softening and flesh gelling as CI symptoms after prolonged cold storage (data not yet published). This results in quality loss and limited market access.

In the present Thesis, 1-MCP, which has been widely reported to alleviate CI of different persimmon cultivars (Salvador *et al.*, 2004a; Woolf & Ben-Arie, 2011), was evaluated as a treatment to alleviate EF-promoted CI during ‘Fuyu’ persimmon cold storage. The mechanism behind the enhanced flesh softening symptom in persimmon by EF-treatment was investigated.

Our results revealed that EF treatments (EF concentrations between 0% and 2% applied for 2 and 4 h) stimulated flesh gelling and fruit softening during cold storage. While fruit softening was not EF dose-dependent, the severity of flesh gelling disorder was observed to be more marked in the fruit treated with the highest EF dose (2% for 2 h and 4 h), which was associated with increased ethylene levels. Treatment with 1-MCP considerably alleviated fruit softening and flesh gelling, and this effect was independent of the EF dose. Thus all the 1-MCP-treated fruits showed similar firmness and flesh gelling incidence values to those of the fruits not submitted to EF treatment. Hence the combined use of 1-MCP and EF treatment is a valuable alternative for the pest control of persimmon fruits, and to preserve fruit quality. Therefore, this treatment met current market access requirements.

The increased ethylene levels observed in the fruits submitted to the highest EF dose suggests that ethylene is implied in promoting CI, fruit softening and flesh gelling. It is known that ethylene is implied in persimmon CI since exogenous exposure aggravates CI (MacRae, 1987; Park & Lee, 2008; Besada *et al.*, 2010a), and ethylene production and the respiration rate are higher in chilling injured fruit after being removed from cold storage (MacRae, 1987; Kader, 2002). The activation of ethylene biosynthesis genes has been related to persimmon softening (Zheng *et al.*, 2005; Pang *et al.*, 2007).

The implication of ethylene in the flesh softening of the EF-treated persimmon fruits was studied by evaluating the expression of ethylene biosynthesis genes, ethylene production and the fruit softening of those fruits submitted, and not submitted, to EF treatment (2% for 4 h) and kept for 14 days at 20°C. Our results showed that EF-stimulated ethylene production by enhancing the expression of biosynthesis ethylene genes, such as *DkACO1*, *DkACO2*, *DkACS2*, *DkERS1* and *DkETR2*; in parallel, the EF-treated fruits drastically lost firmness after 7 days at 20°C, which was not observed in the untreated fruits. Therefore, our results indicated that EF-induced ethylene must trigger fruit softening. Furthermore, the expressions of some genes implied in cell wall degradation, such as *DkEXP3*, *DkPG1*, *DkXTH1* and *DkXTH2*, were also evaluated, but their expressions did not help us to understand the interaction with ethylene levels. Therefore, further studies should be conducted.

IV.3. PHYSIOLOGICAL AND NUTRITIONAL STUDY OF PERSIMMON CULTIVARS INTRODUCED FROM OTHER COUNTRIES IN ORDER TO BROADEN VARIETAL RANGE

IV.3.1. Physiological characterization of fruit maturity and response to deastringency treatment

Currently in Spain, the persimmon crop is based on a monovarietal culture, in which cultivar 'Rojo Brillante' represents 96% of the total persimmon production in Valencia and 83% of total Spanish production (Naval *et al.*, 2010). Thus availability of varieties introduced from other countries with positive agronomic features under Mediterranean conditions and adequate postharvest behaviour would be extremely important to broaden varietal range. In line with this, in the present Thesis the maturity of eight persimmon cultivars introduced from other countries, such as Italy or Japan, which include astringent and non-astringent cultivars, has been physiologically characterised. As astringency removal is a pre-requisite to commercialise astringent cultivars, the effectiveness of deastringency treatment by applying CO₂ under standard conditions (95% CO₂ for 24 h at 20°C) was evaluated in astringent cultivars at harvest.

External skin colour is the non-destructive index currently used for harvesting persimmon fruits; it is considered that fruits have reached the

commercial maturity stage when they display homogeneous orange/red tones. Skin colour evolution in persimmon has been related with internal physicochemical changes during maturity (Salvador *et al.*, 2007; Candir *et al.*, 2009). Accordingly, our results showed that the firmness, soluble tannins and antioxidant capacity of fruits reduced, which ran in parallel to advanced skin colouration. However, the extent of these changes was particular for each cultivar. Accordingly, our results showed that fruit underwent a decrease of firmness, soluble tannins and antioxidant capacity in parallel to the advance of skin coloration. Among the studied cultivars, 'Kaki Tipo' was noted for presenting significantly delayed maturation; that is, when other cultivars reached their homogeneous orange/red tones, 'Kaki Tipo' fruits displayed a pale orange colour, with slightly green areas. In contrast, the maturity of cultivar 'Tone Wase' was quite advanced, although its firmness could be a limiting factor for its commercialisation.

The application of CO₂ treatment to astringent cultivars resulted in all the fruit having a higher acetaldehyde level and lower soluble tannins contents, although the effectiveness of treatment was clearly affected by the cultivar. The sensory panel corroborated that astringency removal was complete in cultivars such as 'Aizumishirazu-A', 'Giboshi' and 'Kaki Tipo', while treatment was not completely effective when applied to cultivars 'Hachiya' and 'Tone Wase'. An effect of maturity stage on treatment efficacy was observed in cultivar Tone Wase, since lack of effectiveness was detected only when treatment was applied to fruits in the most advance maturity stage. This fact can be related to low fruit firmness values since it has been claimed that loss of flesh structure, which fruits suffer in advanced maturity stages, may hinder CO₂ diffusion (Salvador *et al.*, 2008). Therefore the conditions of the CO₂ deastringency treatment need to be optimised for cultivars 'Hachiya' and 'Tone Wase' since treatment duration is a determinant for its efficacy (Besada *et al.*, 2010b). Longer CO₂ exposures must be assayed in both these cultivars.

According to the data obtained, the astringent cultivars introduced from other countries, 'Aizumishirazu-A', 'Giboshi' and 'Kaki Tipo', can be considered alternative cultivars to broaden the current varietal range. 'Kaki Tipo' is particularly interesting because it shows delayed maturation, which may allow the persimmon harvest period to be extended. Non-astringent cultivars 'Hana Fuyu' and 'Jiro' showed a similar maturation date to that of 'Rojo Brillante', but offered the advantage of not needing deastringency treatment. Cultivars 'Hachiya' and 'Tone Wase' should be subjected to further

studies to optimise destringency treatment conditions. It should also be evaluated if the commercialisation of 'Tone Wase' is limited by its low firmness values.

IV.3.2. Nutritional composition of persimmon. Influence of maturity stage and postharvest destringency treatment

The interest of the consumer by healthy properties of fruits has increasingly grown in recent years. Thus the fruit content of the bioactive metabolites that support and promote health has become an added value in the purchase decision of the consumers. Persimmons are considered a good source of health-promoting compounds, such as carotenoids and polyphenols, which play a relevant role in protecting against free radicals and in preventing some human diseases (Rao & Rao, 2007; Gorinstein *et al.*, 2011). In this context, one objective of this Thesis was to study the nutritional composition of persimmon cultivars introduced from other countries, which may be potentially adopted to broaden varietal range. For the purpose of gaining further knowledge about persimmon fruit physiology, our studies approached not only the nutritional composition of fruits in the commercial maturity stage, but also the changes of the main bioactive compounds during maturation. The effect of CO₂ destringency treatment on the nutritional composition of astringent cultivars at harvest was also evaluated.

A first study was conducted to evaluate changes in total and individual sugars, total carotenoids, soluble tannins and total antioxidant capacity, associated with the maturation of ten persimmon cultivars which covered four persimmon types: PVA, PVNA, PCA and PCNA. The results showed that in parallel to an increase in colour and a decline in firmness, which occur during the maturity process, accumulation of total soluble solids, total carotenoids and a slight increase in acetaldehyde production, take place. These changes were characteristic of each cultivar and were not type-dependent. During the maturation process, all the studied cultivars exhibited a decrease of soluble tannins and antioxidant capacity, but the level of both was clearly higher in the astringent cultivars *vs.* non-astringent ones. In fact, the principal component analysis (PCA) applied to the data revealed that soluble tannins and antioxidant capacity were the most relevant parameters for separating non-astringent cultivars (PCNA) from astringent ones (PVA, PVNA, PCA). It is known that

tannins possess strong antiradical activity when they are in soluble form (Gu *et al.*, 2008). However, such activity dramatically decreases when they are in insoluble form. This fact explains that PCNA-type cultivars, in which the concentration of tannins in a soluble form is very low, showed a significantly lower total antioxidant capacity through maturation than that observed in astringent fruits.

On the other hand, the astringency-type had also an effect on the accumulation of individual sugars during fruit maturation. According to Del Bubba *et al.* (2009), sucrose, glucose and fructose were the main sugars found in persimmon flesh. Different trends in the accumulation of these three sugars were observed in astringent cultivars. However the non-astringent ones shared a common sugars accumulation pattern, where sucrose remained at constant values, while glucose and fructose content lowered from the green to colour break stage, to then increase in the commercial stage. Differences in sugar accumulation observed between astringent and non-astringent cultivars might be related to changes in soluble tannins during maturation. It is known that the activity of the enzymes implied in sugar biosynthesis, such as invertase, is affected by gallic and tannic acid (Chen *et al.*, 2002).

In order to complete this study, the content of organics acids (citric, malic and succinic acid) and individual carotenoids (lutein, violoxanthin, zeaxanthin, β -cryptoxanthin and β -carotene), was determined in fruits in the commercial maturity stage. The effect of deastringency treatment on the evaluated bioactive compounds was also investigated since although the effect of this treatment on soluble tannins insolubilisation is well-known (Taira *et al.*, 1998; Salvador *et al.*, 2007; Besada *et al.*, 2012), there is very little information about its influence on other nutritional compounds.

Our study revealed that the total organic acids content in the commercial harvest stage very much depended on the cultivar, and values ranged from 15.8 $\mu\text{g mg}^{-1}$ dw to 30.9 $\mu\text{g mg}^{-1}$ dw. Citric acid, malic acid and succinic acid were the main organic acids found in persimmon, while fumaric acid was present in trace amounts. It is noteworthy that the succinic acid content in non-astringent cultivars was much lower than in the astringent group. Similarly, the total carotenoids content was characteristic of each cultivar, with values as high as 100 $\mu\text{g } \beta\text{-CE } 100\text{g}^{-1}$ dw in cultivar 'Hachiya' and low values, close to 20 $\mu\text{g } \beta\text{-CE } 100\text{g}^{-1}$, in cultivars 'Rojo Brillante' and 'O'goshō'. In all the studied cultivars, β -cryptoxanthin was the predominant carotenoid, which agrees with

that previously reported (De Ancos *et al.*, 2000; Homnava *et al.*, 1990; Kondo *et al.*, 2004; Veberic *et al.*, 2010; Zhou *et al.*, 2011).

Regarding the effect of destringency treatment, our results showed that it led to slight changes in the level of individual organic acids, while total acids content did not change. The effect of this treatment on other nutritional compounds, such as sugars and carotenoids, was stronger and highly dependent on the cultivar. Whereas individual sugars were not affected by treatment in some cultivars, sucrose significantly lowered in others. This could be due to changes in the invertase activity motivated by the tannin insolubilisation process and anaerobic conditions (Borsani *et al.*, 2009; Lara *et al.*, 2011). Likewise in some cultivars like Aizumishirazu-A', 'Giombo' and 'Giboshi', destringency treatment led to an increase in total carotenoids content compared to the values recorded at harvest, while astringency removal did not affect total carotenoids in other cultivars.

For all these reasons, it can be concluded that the nutritional composition of persimmon fruits not only vastly varies among cultivars at harvest, but the effect of destringency treatment on nutritional compounds also depends on the cultivar.

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V. GENERAL CONCLUSIONS

V.1. INVOLVEMENT OF OXIDATIVE STRESS IN PHYSIOLOGICAL DISORDERS OF PERSIMMON FRUIT

- In persimmon fruit (cv. Rojo Brillante), mechanical damage leads to flesh disorders, whose manifestation depends on the level of astringency. 'Flesh browning' is observed only in fruits that have been previously submitted to CO₂ to remove astringency. 'Pinkish bruising', a new described disorder, is manifested in fruits that remain astringent.
- The treatment based on exposing fruits to high concentrations of CO₂ (95% CO₂-24h-20°C), employed to remove astringency from persimmon fruits, leads to an oxidative stress state, observed as increased ROS (H₂O₂ and O₂⁻) levels and as greater activity of ROS scavenging enzymes (SOD, CAT and APX). Moreover, the mechanical damage that fruits are exposed to during packing operations also triggers oxidative stress. This imbalance in the redox state, associated with both CO₂ deastringency treatment and mechanical damage, could lead to a tannins oxidation process, which could result in the manifestation of flesh disorders according to the insoluble/soluble tannins level. Thus flesh browning is correlated to insoluble tannins, while pinkish-bruising is correlated to high soluble tannins content.
- The comparative study of astringent and non-astringent cultivars revealed that fruit sensitivity to the flesh disorders associated with mechanical damage is clearly influenced by persimmon type. Similarly to 'Rojo Brillante', other astringent cultivars manifested browning and/or pinkish-bruising, associated with mechanical damage depending on the soluble/insoluble tannins level. Non-astringent cultivars did not exhibit the browning/pinkish-bruising associated with mechanical damage, but mechanical damage in these cultivars resulted in uncoloured bruised flesh areas.
- Low-temperature storage of 'Rojo Brillante' persimmon leads to gradual oxidative stress, which is not alleviated by 1-MCP. The manifestation of CI symptoms, which occurs when fruits are transferred from low to moderate temperatures, is associated with oxidative burst, with major H₂O₂ accumulation, and also with a sharp increase in the activity of CAT, POD, and LOX. The reduction of CI symptoms by the 1-MCP treatment is linked to lower POD activity levels and enhanced CAT enzyme activity during shelf-life period, which result in reduced H₂O₂ accumulation.

IV.2. EVALUATION OF POSTHARVEST TREATMENTS TO IMPROVE FRUIT QUALITY DURING COLD STORAGE

- The effect of applying a controlled atmosphere to prolong persimmon fruit was highly dependent on the cultivar. The combination of CA based on 4-5% O₂+ N₂ and a 1-MCP pretreatment substantially alleviated chilling injury symptoms (flesh softening and gelling) in cultivar 'Triumph', and helped prolong the storage period up to 3 months. For cultivar 'Rojo Brillante', although CA storage also retarded fruit softening and alleviated flesh gelling, internal flesh browning associated with CA was the main storage limiting factor. The use of CA has a positive effect on retarding the decay development caused by *Alternaria alternata* in both cultivars 'Rojo Brillante' and 'Triumph'.
- The use of short-term high CO₂ treatments (95% for 12-36 h) applied before prolonged storage may be considered an alternative technology to alleviate CI symptoms during the storage of non-astringent cultivar 'Fuyu'. This treatment alleviates flesh gelling by preserving the integrity of cell walls and the plasmalemma.
- Postharvest disinfection treatment with ethyl formate leads to significant fruit softening and flesh gelling in relation to CI in persimmon fruits after prolonged cold storage. Such disorders are markedly alleviated by 1-MCP pretreatment. Therefore, the combined use of 1-MCP and the ethyl formate treatment is a valuable alternative for the pest control of persimmon fruits, while preserving fruit quality.
- Ethylene is implied in promoting CI, fruit softening and flesh gelling, and is associated with ethyl formate treatment since the relative expressions of ethylene biosynthesis genes and ethylene production increased in ethyl formate-treated fruits.

IV.3. PHYSIOLOGICAL AND NUTRITIONAL STUDY OF PERSIMMON CULTIVARS INTRODUCED FROM OTHER COUNTRIES IN ORDER TO BROADEN VARIETAL RANGE

- Of all the evaluated cultivars introduced from other countries, 'Aizumishirazu-A', 'Giboshi' and 'Kaki Tipo', can be considered alternative astringent cultivars to help broaden the current varietal range. Non-astringent cultivars 'Hana Fuyu' and 'Jiro' offer the advantage of not needing deastringency treatment.
- The nutritional study of ten persimmon cultivars from the four persimmon types (PVA, PVNA, PCA and PCNA) in different maturity stages revealed that the soluble tannins content and total antioxidant capacity decline as the maturity process advances, while total soluble solids increase and accumulation of individual sugars take place. All these changes were affected by astringency type. The cultivars that belong to the non-PCNA type exhibit a higher soluble tannin content and greater antioxidant capacity than the PCNA cultivars during the maturation process. Besides, the PCNA-type cultivars share a common sugar accumulation pattern, while the non-PCNA cultivars exhibit different trends.
- During maturation, all the cultivars exhibited a decline of firmness, and also an increase of flesh carotenoids and acetaldehyde production. Such changes were cultivar-dependent rather than type-dependent.
- The high CO₂ deastringency treatment applied to astringent cultivars, besides the decline of soluble tannins content and antioxidant capacity, induces changes in individual sugars, organic acids content and carotenoids composition. The extent of such changes is highly cultivar-dependent.

