Development of a new strategy based on the application of phytoregulators to induce phenolic acids in olive fruits

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ABSTRACT

A new strategy based on the application of phytoregulators was developed to minimize the natural degradation of antioxidants during olives storage. For that purpose, the effect of the phytoregulators abscisic acid and methyl jasmonate on olive phenolic acids was studied. In particular, four treatments were applied: abscisic acid was applied as a paste and as a spray together with low temperatures; whereas methyl jasmonate as a paste at low temperatures and as a vapor at mild temperatures. As a result, abscisic acid spray and methyl jasmonate vapor resulted in significantly higher contents of all phenolic acids. In contrast, when the phytoregulators were applied in paste form, no effect was observed. To confirm these results, the IC50 value was also determined. An increase of IC50 from 2.31 to 4.10 µg/ml after abscisic acid spray treatment and from 2.72 to 5.36 µg/ml after methyl jasmonate vapor treatment was obtained.

1. Introduction

Olea europaea L. is a typical tree widely cultivated for oil and table olive production in the Mediterranean Area because of their pharmacological properties (Visioli, Poli, & Galli, 2002). The beneficial effects of olives have been attributed to a convenient fatty acid profile and to the presence of certain phenolic compounds (Ryan & Robards, 1998). Phenolics are minor constituents regarded as relevant contributors not only to the health promoting properties of olives but also to their typical taste and oxidative stability (Coni et al., 2000; Reboredo-Rodríguez et al., 2017). Together with oleuropein and hydroxytyrosol, phenolic acids are one of the most important phenolics in olives (Arslan & Özcan, 2011; Romero, García, Brenes, García, & Garrido, 2002). They have been largely described in the literature as potent food antioxidants (Ferreira, Barros, & Abreu, 2009).

However, phenolic acids, as most phenolics, are frequently lost during olives storage as a result of chemical degradation. Actually, reductions of up to a third of phenolic content have been reported in olives after short storage periods (Benito, Oria, & Sánchez-Gimeno, 2009). This reduction is associated with a number of additional changes also occurring in olives as a result of storage. Among them, loss of volatiles, alterations in chemical quality indices and FA composition, appearance of ‘off-flavor’, etc. (Inarejos-García, Gómez-Rico, Desamparados Salvador, & Fregapane, 2010; Kalua, Bedgood, Bishop, & Prenzler, 2006). For this reason, it is necessary to develop new strategies that enable olive phenolics to be preserved, particularly when delay in oil processing occurs.

On the other hand, in the last few years, investigations have proved the possibility of improving the sensorial and nutritional characteristics of based plant foods exposed to phytoregulators. Many natural phytohormones have been used as phytoregulators; among them, abscisic acid (ABA) and methyl jasmonate (MJ) are some of the most used (Huang et al., 2016). ABA is defined as a stress plant hormone because of its rapid accumulation in response to stress whereas MJ acts as a triggering molecule to...
stimulate the production of secondary metabolites. The effect of ABA on the accumulation of phenolics has been largely reported (Cantín, Fidelibus, & Crisosto, 2007; Huang et al., 2016; Liang et al., 2013; Peppi, Fidelibus, & Dokoozlian, 2006). Similarly, MJ has been demonstrated to be effective in increasing the concentration of phenolics acids in various plant foods other than olives (Heredia & Cisneros-Zevallos, 2009; Horbowicz, Chrzanowski, Koczokdaj, & Mitrus, 2011; Kim, Fonseca, Choi, & Kubota, 2007).

For example, an increase of caffeic acid from 10.0 to 30.0 mg has been reported after ABA treatment of *Salvia miltiorrhiza* (Liang et al., 2013). In the same way, an increase of caffeic acid from 11.2 to 22.7 mg and from 12.9 to 228 mg after MJ treatment has been estimated in romaine lettuce (Kim et al., 2007). The effect of MJ treatment on berries and seeds phenolic composition has been extensively studied (Flores, Blanch, & Ruiz del Castillo, 2015; Flores & Ruiz del Castillo, 2015, 2016). More recently, it has been reported the effectiveness of MJ vapor to induce phenolic acids in olive fruits. For example, an increase of caffeic acid from 4.72 to 11.57 mg kg\(^{-1}\) was estimated after MJ treatment (Flores, Blanch, & Ruiz del Castillo, 2016). Although the results obtained were satisfactory, some drawbacks were found. First, MJ is costly in such a way that the use of high amounts is not economically profitable. Second, MJ is a very active phytoregulator which makes it difficult the selection of the adequate concentration to obtain the desired effect.

Our aim now is to search for alternative strategies to the application of exogenous phytoregulator namely ABA and MJ under different conditions that preserve phenolic acids content during olive storage on the one hand, and overcome the limitations of the MJ vapor on the other hand. MJ was applied to the olives as a paste in combination with low temperatures and as a vapor in combination with mild temperatures whereas ABA was applied as a paste and as a spray together with low temperatures. The results obtained from these treatments were compared with those obtained from the study of MJ vapor on olive phenolic acid (Flores et al., 2016).

2. Materials and methods

2.1. Samples and chemicals

HPLC-grade acetonitrile was obtained from Lab Scan (Dublin, Ireland). HPLC-grade MeOH and EtOH was supplied by WVR Inc. (Bridgeport, PA, USA). Ultrapure water was collected from a purification system (Millipore Milford, MA, USA). ABA was purchased from Across Organics (New Jersey, USA). Phenolic acid (i.e. gallic, vanillic, p-coumaric, caffeic, chlorogenic and ferulic acid) and 1,1-diphenyl-2-picrylhydrazil (DPPH) standards were supplied by Sigma (Steinheim, Germany). Olive fruits (*Manzanilla cultivar*) were hand-picked from the trees in December 2015 in Cáceres (Spain). Only undamaged fruits without any kind of infection or physical injury were selected for the experiments. All fruits exhibited approximately the same size and maturity stage. After harvesting, the olive fruits were immediately subjected to the application of phytoregulators, as explained below.

2.2. Application of phytoregulators

Approximately 960 g of olive fruits were weighed for the experiments. Based on the use of ABA and MJ as phytoregulators, different strategies were tested: ABA in paste form, MJ in paste form and ABA in spray form. Additionally, MJ in vapor form was also applied and used as a reference. On the basis of our previous experience, all phytoregulators were applied in combination with low temperatures except MJ vapor, which was applied at mild temperature (Flores et al., 2016; Ruiz del Castillo, Flores, & Blanch, 2010).

2.2.1. Application of ABA or MJ paste

A 240-g weight of olives was placed in a 1 L round glass container with 25 cm diameter and a plastic lid. The headspace volume on the top of the samples was approximately half of the whole volume of the container. Samples were treated with 48 mg of ABA or MJ (0.2 mg g\(^{-1}\) olive fruit) in Carbowax 20 M paste, which was prepared by mixing 10 g Carbowax 20 M in 20 ml distilled water (1/2, w/v). This emulsion was homogeneously applied by using a spatula over the olives. At the same time, another 240 g of olives were placed in other similar glass container and treated with Carbowax 20 M paste alone (i.e. without phytoregulator) to be used as a control. Both containers control and treatment were stored at 4°C for 30 days. After that time, the paste was carefully removed from the olives using a dry paper sheet. Then, all olives included in the study were used for the extractions and aliquots of them were used for the analyses.

2.2.2. Application of ABA spray

A 240-g weight of olives was placed in a glass container and treated with 48 mg of ABA (0.2 mg g\(^{-1}\) olive fruit) in 0.05% Tween-20 mixture, which was prepared by mixing 70 μl Tween-20 in 150 ml distilled water. The treatment was carried out by applying a spray to runoff over the olives. Simultaneously, other 240 g of olives were also placed in other glass container and sprayed with 0.05% Tween-20 mixtures alone (i.e. without ABA) to be used as a control. Both containers, controls and treatments, were also stored at 4°C for 30 days.

2.2.3. Application of MJ vapor

Based on our previous experience (Flores et al., 2016; Flores, Pérez, Gil, Blanch, & Ruiz del Castillo, 2013), MJ vapor was applied to the olive samples included in this study.

In the four treatments tested, the cores of control and treated samples were removed and the pulps were analyzed. The samples were immediately frozen at −80°C until analysis.

2.3. Analysis of phenolic acids in all treated olive samples

2.3.1. Extraction of phenolic acids

Phenolic acid composition was examined in olive fruits untreated and treated by all the strategies applied. The extraction was performed on the basis of the method elsewhere described (Vinha et al., 2005) with slight modifications. First, a 60 ml volume of 80:20 (v/v) methanol:water was added to a 5 g weight of sample. After homogenization and centrifugation, 30 ml of hexane was added to the resulting extract to eliminate the remaining oil. Once discarded the hexane layer, the methanolic extract was collected, filtered and analyzed by HPLC as detailed below. Extractions of each single sample including untreated-controls and olives treated were accomplished in duplicate.

2.3.2. HPLC analysis of phenolic acids

A Konik-Tech model 560 (Barcelona, Spain) liquid chromatograph fitted with a manual injection valve (model 7725i,
Konik-Tech, Barcelona, Spain) and having a 20 μl sample loop was used for the analyses. The experimental conditions used were those earlier optimized (Flores & Ruiz del Castillo, 2016). Chromatograms were recorded at two different wavelengths. Gallic acid and vanillic acid were detected at 280 nm, whereas caffeic, p-coumaric, ferulic and chlorogenic acids were measured at 320 nm. Blanks between consecutive runs were performed to assure the washing of the equipment. Three HPLC runs were performed for each single extract. Stock solutions of the standard compounds were prepared in 70% (v/v) methanol to final concentration of 1 mg ml\(^{-1}\). Each stock solution was further diluted to obtain six concentrations of the standard to perform the calibration curves. Peak areas for the extracts and standards were integrated by use of Konikrom Plus (KNK-725–240).

### 2.4. DPPH assay in ABA spray and MJ vapor-treated olives

The free radical scavenging activity was assayed in controls and olives treated with ABA spray and MJ vapor as follows:

#### 2.4.1. Extraction

Phenolic compounds from control and olives treated with ABA spray and MJ vapor were extracted by following the analytical procedure described elsewhere (Shin et al., 2008). In brief, 10 g weight of sample was homogenized for 3 min with 10 ml of 80% acetone at 4°C using a coffee grinder. The resulting mixture was filtered through No. 1 Whatman paper and the acetone was evaporated off by using a rotary evaporator at 45°C. The dry extracts were brought to 5 ml MeOH for the DPPH assay.

#### 2.4.2. Determination of DPPH activity

The ability of the extracts to scavenge DPPH* radicals was performed according to a slight modification of the method developed by Smith, Reeves, Dage, and Schnettler (1987). Each extract was further diluted to final concentrations of 15.6, 62.5, 125, 250 and 500 μg ml\(^{-1}\) before being transferred to a 96-well microtiter plate. Each extract solution before adding DPPH was used as a blank. Each well contained 50 μl of the sample and 150 μl of DPPH (400 μM). Decrease of absorbance, with respect to DPPH solution measured immediately, was monitored at 517 nm after 30 min of incubation at 37°C. The percentage inhibition of the DPPH by each dilution of samples was calculated considering the percentage of the steady DPPH in solution after reaction. The results were expressed as the concentration of extracts that gives rise to a 50% reduction in the DPPH (i.e. IC\(^{50}\) values). The experiments were performed in duplicate for each sample.

#### 2.5. Statistical analyses

Analysis of variance of data on the influence of phytoregulators on the content of phenolic acids and DPPH activity in olive fruits was performed using JMP Statistics software package version 8 (purchased from SAS Institute Inc., NC, USA). The effect of the application of ABA and MJ was assessed by the Fisher test. Differences between data were compared by least significant differences. The values used were always the mean of the three replicates performed. Differences at \( p \leq 0.05 \) were considered to be significant.

### 3. Results and discussion

The results obtained from the different strategies applied were comparatively studied. Table 1 represents phenolic acid contents (mg 100 mg\(^{-1}\) weight ± standard deviation) in olive fruits treated by applying different strategies: ABA paste, ABA spray, MJ paste, all of them at low temperature, and MJ vapor at mild temperature after 30 day storage. The results obtained from untreated-control olive samples corresponding to each single treatment are also included for comparison. In general, the concentrations of phenolic acids obtained in the untreated-control olives for each treatment were always in the same range. Just negligible differences were estimated among them which can be largely attributed to the natural variability in the chemical composition of olive fruits. Factors contributing to phenolic content variation between olive fruits include climate, position on the tree, rootstock and agricultural practices (Vinha et al., 2005). It is also interesting to point out that the levels in all control samples were slightly lower than those reported in the literature for olives analyzed right after hand-picked (Arslan & Özcan, 2011). This variation is associated with the chemical decomposition of phenolic acids with the time (Benito, Oria, & Sánchez-Gimeno, 2009). In fact, inappropriate storage of olive fruits including long periods result in phenolic contents reduction and other quality parameters (Nergiz & Unal, 1991).

As also seen in Table 1, the application of ABA paste at low temperatures did not affect significantly (\( p > 0.05 \)) the

### Table 1

| Samples          | Gallic acid | Chlorogenic acid | Vanillic acid | Caffeic acid | p-Coumaric acid | Ferulic acid |
|------------------|-------------|------------------|---------------|--------------|-----------------|--------------|
| ABA paste control| 0.20 ± 0.02a| 0.84 ± 0.07a     | 0.93 ± 0.03a  | 0.43 ± 0.08a | 0.11 ± 0.07a    | 0.35 ± 0.06a  |
| ABA paste treated| 0.23 ± 0.08a| 0.81 ± 0.06a     | 1.03 ± 0.07a  | 0.53 ± 0.02a | 0.09 ± 0.05a    | 0.33 ± 0.03a  |
| ABA spray control| 0.18 ± 0.07a| 0.90 ± 0.05a     | 0.89 ± 0.06a  | 0.47 ± 0.06a | 0.13 ± 0.02a    | 0.42 ± 0.05a  |
| ABA spray treated| 0.53 ± 0.09b| 1.58 ± 0.08b     | 1.81 ± 0.10b  | 1.50 ± 0.07b | 0.49 ± 0.07b    | 0.98 ± 0.05b  |
| MJ paste control | 0.13 ± 0.01a| 0.98 ± 0.04a     | 0.82 ± 0.02a  | 0.38 ± 0.07a | 0.16 ± 0.04a    | 0.45 ± 0.04a  |
| MJ paste treated | 0.19 ± 0.07a| 0.86 ± 0.05a     | 0.68 ± 0.05a  | 0.39 ± 0.01a | 0.05 ± 0.04a    | 0.38 ± 0.02a  |
| MJ vapor control | 0.13 ± 0.07a| 0.92 ± 0.05a     | 0.96 ± 0.06a  | 0.51 ± 0.06a | 0.09 ± 0.02a    | 0.40 ± 0.05a  |
| MJ vapor treated | 0.63 ± 0.09b| 1.62 ± 0.08b     | 1.69 ± 0.10b  | 1.25 ± 0.07b | 0.53 ± 0.07b    | 1.04 ± 0.05b  |

Different letters in the same column between control and treated samples within each single treatment indicate significant (\( p < 0.05 \)) differences.
phenolic acid composition in olives. As an example, chlorogenic acid content was 0.84 mg 100 mg$^{-1}$ in control and 0.81 mg 100 mg$^{-1}$ in treated samples, caffeic acid content was 0.43 mg 100 mg$^{-1}$ in control and 0.53 mg 100 mg$^{-1}$ in treated and p-coumaric acid content was 0.11 mg 100 mg$^{-1}$ in control and 0.09 mg 100 mg$^{-1}$ in treated. Our results suggest that the application of ABA in paste form is not effective and, therefore, this procedure was discarded.

As also seen in Table 1, the application of ABA spray at low temperatures, contrary to ABA paste, did have significant ($p < 0.05$) effect on phenolic acid contents. Specifically, gallic acid increased from 0.18 to 0.53 mg 100 mg$^{-1}$, chlorogenic acid from 0.90 to 1.58 mg 100 mg$^{-1}$, vanillic acid from 0.89 to 1.81 mg 100 mg$^{-1}$, caffeic acid from 0.47 to 1.50 mg 100 mg$^{-1}$, p-coumaric acid from 0.13 to 0.49 mg 100 mg$^{-1}$ and ferulic acid from 0.42 to 0.98 mg 100 mg$^{-1}$. These results confirm the relevance of the procedure used to apply exogenous ABA. An accumulation of phenolic compounds in general (Sandhu, Gray, Lu, & Gu, 2011) and phenolic acids in particular (Liang et al., 2013) in plant base foods, other than olives, treated with ABA as a spray (Sandhu et al., 2011; Xi et al., 2013) and dissolved in ethanol (Liang et al., 2013) has been observed. It is postulated that the induction of phenolic acids by ABA spray is due to the influence of ABA on enzymes involved in the phenylpropanoid pathway, which is responsible for the bioformation of most phenolics including phenolic acids. Phenylpropanoid pathway is initiated by phenylalanine ammonia-lyase (PAL) enzyme. Further in the pathway, flavonoids including phenolic acids are formed by the action of different specific enzymes. It is believed that ABA spray activates PAL enzyme regulating the beginning of the pathway whereas when applied as a paste no impact on PAL activity is achieved.

When MJ has been applied as a vapor, promoting effect on the production of phenolic acids has been widely demonstrated by other authors (Gumerova, Akulov, & Rumyantseva, 2015; Ying, Peng, Shouhong, & Lei, 2009) and by ourselves (Flores & Ruiz del Castillo, 2016). However, exogenous MJ application as a paste has been successfully used to enhance volatiles in potato tubers (Ruiz del Castillo et al., 2010) and modify fatty acid fraction in tomato fruits (Czapski, Horbowicz, & Saniewski, 1992). In both cases, the temperature applied during the procedure was similar to that here used. The unsuccessful results obtained in this study from the application of the phytoregulator, both ABA and MJ, as a paste (values shown in Table 1) might be due to the difficulty to penetrate olive skin. The reason why paste gets absorbed properly into the tuber and tomato whereas it did not enter through olive skin is not well understood. It is hypothesized that larger fruits possess larger skin surface favoring the application and, therefore, the absorption of the phytoregulator. Other possibility might be the thicker olive skin as compared with those of potato and tomato. All in all, when ABA or MJ is applied to olive fruits, the use of the phytoregulator as a paste over the sample was ruled out.

Additionally, results show a significant increase in phenolic acids contents in olive fruits treated with MJ vapor (Table 1). Similar results have been already published in the literature in plant foods other than olives such as Salvia miltiorrhiza, berries and wheat (Flores & Ruiz del Castillo, 2016; Gumerova et al., 2015; Ying et al., 2009). The results obtained from MJ vapor application at 25°C as compared with those provided by MJ paste at 4°C support the theory about the difficulty of absorption of the phytoregulator when applied to the olives as a paste.

By comparing the application of ABA spray and with MJ vapor (see Table 1) the increments in phenolic acid contents were similar. These results reflect that both phytoregulators are equally applicable as stimulating agents in inducing phenolic acid production in olives as long as each one is applied to the right temperature. In this line, it is deduced that MJ vapor, equally to ABA spray, penetrates olive skin, accumulates inside the fruit and enhances the production of phenolic acids by promoting PAL activity. Although the impact of phytoregulators on PAL enzyme has not been, to date, specifically studied in olive fruit, promoting effect of certain natural phytohormones has already been described in other plant-derived foods (Flores, de la Peña Moreno, Blanch, & Ruiz del Castillo, 2014; Kim et al., 2007).

With a view of evaluating the possible correlation between the increase in the phenolic acid content and the antioxidant activity, $IC_{50}$ values obtained from ABA spray and MJ vapor treatments were determined and compared with controls. Table 2 indicates the $IC_{50}$ values of olives untreated and treated with ABA spray and MJ vapor after 30-day storage. As seen in the table both control samples exhibited a similar $IC_{50}$ (i.e. 2.31 µg ml$^{-1}$ for ABA spray control and 2.72 µg ml$^{-1}$ for MJ vapor control). In the same way, significant ($p < 0.05$) increases in $IC_{50}$ as a result of the application of phytoregulators were observed for both ABA and MJ. In particular, an increase from 2.31 to 4.10 µg ml$^{-1}$ was measured after the application of ABA spray whereas the $IC_{50}$ increased from 2.72 to 5.36 µg ml$^{-1}$ for olives treated with MJ vapor. From these results, the increase observed in the content of phenolic acids after olives exposition to ABA spray and MJ vapor is not proportional to the trend of the free radical scavenging activity, this result could be explained by the fact that ABA spray and MJ vapor affect negatively components other than phenolic acids present in the extract, which are able to decrease the overall antioxidant activity. This hypothesis is being currently studied. The effect of MJ vapor on $IC_{50}$ has been widely reported in a number of plant foods (Flores et al., 2015, 2013; Li et al., 2017). On the contrary, the results obtained from ABA spray are scarce in the literature (Huang et al., 2016; Liang et al., 2013).

In olives, the phenolic acid content, as other antioxidants, serves as an important quantitative parameter due to its correlation with the peroxide number, FFA and sensorial quality (Gramadzka & Wardencil, 2011). As reported, the existence of high content of phenolic acids contributes to the oxidative stabilization of fatty acids and peroxides and, as a result, to extend olive fruit self-life. In addition, the production of olive oil and table olives involve extraction and/or chemical

| Table 2. DPPH scavenging activity expressed as $IC_{50}$ (µg ml$^{-1}$) of olives untreated and treated with ABA spray at low temperature and MJ vapor at mild temperature after 30-day storage.

| DPPH activity     | ABA spray control | ABA spray treated | MJ vapor control | MJ vapor treated |
|-------------------|-------------------|-------------------|------------------|------------------|
| $IC_{50}$ (µg ml$^{-1}$) | 2.31 ± 0.07a | 4.10 ± 0.05b | 2.72 ± 0.06a | 5.36 ± 0.06b |

The values are expressed as means ± SD ($n = 6$). Different letters between control and treated indicate significant ($p < 0.05$) differences.
treatment of the fruit which impacts on the phenolic acid content, particularly for caffeic acid. Natural occurrence of high phenolic acids contents in the starting olive fruit guarantees sufficient antioxidant content to assure the final product quality.

It is necessary to emphasize that phenolic acid contents found in this study in olives treated with phytoregulators whatever the strategy used were always lower than the values reported in the literature for olive fruits (Arslan & Özcan, 2011). This was somehow expected considering that the measurements were performed after prolonged storage. In this respect, it is important to bear in mind that the purpose of this study was not to obtain particularly high content of phenolic acids but avoid the natural decline of phenolic acids observed during olives storage.

As a conclusion, the postharvest exposition to ABA spray or to MJ vapor allows olive fruits to be stored for at least 30 days without loss either in phenolic acid content. The applications of ABA or MJ as a paste were however unsuccessful. The reported antioxidant power of phenolic acids dampens the autogeneration of peroxides and stabilizes free fatty acids, delaying the onset of oxidation and rancidity and therefore increases oil shelf life. In addition, the use of natural phytohormones that enable plant defenses to be regulated is always recommendable rather than the use of biocides. The postharvest application of ABA spray is therefore proposed as a more economical alternative to MJ vapor to increase phenolic acids content in the starting olive fruits. The next step is now to evaluate the influence of different concentrations of ABA spray and MJ vapor not only on the phenolic acid content but also on other olive phenolics with a view to getting an insight into the effect on IC50 here observed. In addition, the preharvest application of phytoregulators to improve olive oil quality is also in progress.

Disclosure statement
No potential conflict of interest was reported by the authors.

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