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Abstract

Calcium is largely used in agri-food industry due to its positive effects, such as, preserving activity or protection against pathogens. An increase in firmness and phenolic content has been spotted after post-harvest spraying of calcium chloride ($CaCl_2$) solutions. The aim of this research was to investigate the effects of pre-harvest applications of two $CaCl_2$ concentrations (Ca 0.5% and Ca 1.0%) during the Spanish-style elaboration process (beginning, middle and final of fermentation process). In particular, the Ca concentration in the whole fruit and in the different tissues (pulp, pit and seed), the firmness, the phenolic content, and the antioxidant activity were analysed in 'Ascolana tenera' table olive. An increase in Ca concentration in the pulp (20.2% and 55.1%) and in the seed (13.4% and 36.5%) was observed along monitoring period when 0.5% and 1.0% $CaCl_2$ concentrations were respectively compared with Ca 0% (water). No significant Ca increase was found in the pit. The fruit firmness was higher after the Ca-treatments (22.8% and 68.3%, respectively). The content of Hydroxytyrosol, Tyrosol and Verbascoside decreased during the elaboration process. The highest content found of these phenols were at the first date of monitoring for 1.0% $CaCl_2$ concentration. Hydroxytyrosol, Oleuropein and PB1 showed significant difference at different monitoring dates. During the fermentation period, the antioxidant activity presented an increase between 63.3 and 87.2% with respect to Control (Ca 0%). Similar increasing trend was also registered for the phenolic compounds. Our results are useful for the table olive sector to improve the handling in post-harvest processing and increase the final quality of the olives.

Keywords: 'Ascolana tenera'; post-harvest; firmness; phenolic compounds; antioxidant activity.

Introduction

Farmers growing olive trees (*Olea europaea* L.) in the Mediterranean basin are improving the production processes to increase the quality of the table olives on the international markets (International Olive Council, 2020). At the industrial level, there are different production methods, and the Spanish-style is one of the most important used all over the world (Rallo et al., 2018). Olives are debittered with lye solutions and then washed and submitted to lactic fermentation in brine for several months. Different table olive varieties are processed according to the Spanish-style such as 'Manzanilla', 'Gordal', 'Hojiblanca', 'Carrasqueña' and 'Cacereña' (Cabrera-Bañegil et al., 2018). Also 'Ascolana tenera' is processed as the Spanish-style. This olive variety, grown mainly in the Marche Region in the central Italy under the Protected Origin Designation "Oliva Ascolana del Piceno", has a big-size fruit and a high pulp-to-pit ratio. However, it has a very sensitive pulp and the fruits can be subjected to damages during harvest, transport and processing, decreasing the final quality and value of the production (Lodolini et al., 2019; Morales-Sillero et al., 2021).

Calcium has an important role in the fruit quality (Conway et al., 2002; Morales-Sillero et al., 2021) by creating cross-bridges with pectic polymers in the cell wall strengthening it (Manganaris et al., 2005), and increasing its resistance to hydrolytic enzymes (Miceli et al., 1999). Calcium is also able to improve the resistance against pathogens, such as Anthracnose or Botrytis, affecting fruit tissues (Miceli et al., 1999; Langer et al., 2019; Madani et al., 2014). The decrease in calcium content leads to enzymatic browning of the tissues (Jemrić et al., 2016) , causing the degradation of the fruit and its quality decline. Ca-treatments has been used in agri-food industry to improve the quality of many fruits, such as, apples (Holb et al., 2012), olives (Gouvinhas and Barros, 2021; Tsantili et al., 2008), peaches (Manganaris et al., 2005) and table grapes (Ciccarese et al., 2013). Pre-harvest treatments applied to olives, using foliar application of calcium, demonstrated that a higher calcium content augmented the phenolic compounds and increased the fruit firmness, slowing down the degradation of the cell wall (Morales-Sillero et al., 2021). Post-harvest Ca-treatments may be useful as well to enhance the firmness and elongate the shelf life of the olive (Rincón and Martínez, 2015). In the same way, spraying calcium solution to the fruits after harvest may be useful to overcome calcium deficiencies. The bibliography also reports that fruits, in this case the table olives, tend to form cracks in the skin surface (membrane breaking) when they are mature, (Harker and Ferguson, 1988). These fractures can act like an easy-way access for the calcium into the fruit. However, the fruit quality enhancement induced by calcium, as well as its absorption mechanisms, transport and storing in the fruit itself, is not yet fully understood (Hocking et al., 2016).

Previous studies highlighted that there may be difference in the Ca concentration required to increase its content in the fruit based on the tree species or cultivar (Lara, 2013). Tomatoes increased the firmness when dipped in a $CaCl_2$ (2%) solution after harvest (Gharezi and Gharezi, 2012), as well as papayas when treated with 2.5% concentration (Gao et al., 2020) or fresh-cut cantaloupe melons when dipped in $CaCl_2$ (1-5%) concentration (Luna-Guzmán et al., 1999). Post-harvest treatments with $CaCl_2$ increased the total phenolic content for storage cherries, along with an improvement of the antioxidant activity (Aghdam et al., 2013). In previous research studies, post-harvest Ca-treatment effects have been assessed in Californian-style black and green table olives (Martín-Vertedor et al., 2021, 2020).

The effects of pre-harvest application of calcium chloride on the quality of Spanish-style table olives have not been deeply investigated. The lack of literature about this topic can depend on several factors, as for example the effective concentration or the frequency of application to be applied in order to obtain significant changes in the fruit characteristics (Morales-Sillero et al., 2021). For all the above, the proposed hypothesis states that pre-harvest Ca applications increase table olives quality after the elaboration process. In order to contrast this, the aim of this study was to deepen the knowledge about the effect of two $CaCl_2$ concentrations applied in three different dates of the fruit development (Morales-Sillero et al., 2021), through the quality of the olives during the Spanish-style fermentation process for table olives.

Material and Methods

Chemical reagents

To apply the foliar calcium treatments in the olive trees, $CaCl_2$ $2H_2O$, 99.0% (Labkem, Barcelona, Spain) and a commercial adjuvant Tween-20, 0.5% (ThermoFisher Scientific, MO, USA) were used. For the olive elaboration according to the Spanish-style, sodium hydroxide, acetic acid and sodium chloride were purchased from Sigma Aldrich (Sigma-Aldrich, San Luis, USA). Commercial grade nitric acid (Sigma-Aldrich, San Luis, USA) solutions were used for calcium analysis. For phenol profile apigenin-7-O-glucoside, hydroxytyrosol, luteolin, oleuropein, procyanidin B1 (PB1), and verbascoside were purchased by Extrasynthése (Genay, France). Apigenin, epicatechin, luteolin-7-O-glucoside, quercetin-3-rutinoside and tyrosol (p-HPEA) by Sigma-Aldrich Chemie (Steinheim, Germany). p-coumaric acid by Fluka Chemie (Steinheim, Germany). Acetonitrile and methanol (HPLC grade) were supplied from Fisher Scientific (Loughborough, UK), formic acid by PANREAC (Barcelona, Spain), and sodium fluoride by Sigma-Aldrich Chemie (Steinheim, Germany).

Plant material

The study was carried out in 2017 on the cultivar ‘Ascolana tenera’ in a drip-irrigated olive orchard planted in 2009 in Montalto delle Marche (Central Italy) with a planting density of 200 trees ha^{-1} (7.0 m × 7.0 m) as reported in Morales-Sillero et al. (2021). The cultivar was drip-irrigated orchard (~1500 $m^3 \cdot ha^{-1}$). The mean temperature and total annual rainfall were 13.4 °C and 820 mm, respectively (Servizio Agrometeo ASSAM, Regione Marche).

This variety is characterized by a large size fruit (7-8 g), a high pulp-to-pit ratio, and a very soft flesh. The high sensitivity of 'Ascolana tenera' can lead to the appearance of black spots on the skin during the harvesting operations and the transport process. The fruit is typically picked at the pale green ripening stage for small-scale production (local market) of table olives under the Protected Designation of Origin 'Oliva Ascolana del Piceno' (Lodolini et al., 2019).

During our study, full bloom and harvest occurred on May 30th and September 14th 2017, respectively. Olives were harvested at yellowish-green skin/pulp colour stage of maturation and presented morphological characteristics as indicated in Morales-Sillero et al. (2021). The fruit mass of 'Ascolana tenera' variety in all the experimental treatments was 5 g.

Experimental Design

Two different calcium concentrations, Ca 0.5% and Ca 1.0%, were administered in the morning by foliar applications with a commercial adjuvant (Tween-20; 100 $mL \cdot 100\ L^{-1}$). The treatments were repeated three times during the fruit development: i) at the end of fruit set; ii) at the end of pit hardening; and iii) two weeks before harvesting. Water was used as Control (Ca 0%) and sprayed the same days when calcium was applied. Each calcium concentration was applied to three replicates of five homogeneous trees in a randomized block design with guard trees around the trees, as reported in Morales-Sillero et al. (2021).

Spanish-style elaboration process

Immediately after harvest, the olives were kept separated according to the Ca-treatment, transported to the processing facility and separately elaborated according to the Spanish-style (Schaide et al., 2019). Each replicate of fermented fruits consisted of three 225L-fermentors (olives of 5 trees) for ~110 kg of olives per replica. Thus, olives were treated with NaOH solution (2.5%, w/v at 25 °C) for debittering until 2/3 of the flesh was reached. Then the alkaline solution was removed and clean water was flown for 12 hours for the washing stage. Finally, to allow the fermentation process, olives were placed into NaCl brine solution at 10%, w/v that was stabilized with olives at 6% of NaCl. The total chloride concentration present in the brine was also monitored and controlled. The pH of the brine was continuously adjusted a 3.8 point in all the trials by adding lactic acid, in order to maintain adequate chemical condition in each fermenter. A spontaneous fermentation occurred between October 2017 and March 2018 (160 days). The final of the fermentation process was also determined by evaluating olive color by the tasting panel. According to official protocol of the Protected Designation of Origin 'Oliva Ascolana del Piceno', after the elaboration process and during the conservation period, NaCl concentration was maintained around 8% and the pH below 4.5. No enterobacteria, coliforms, *Bacillus*, and *Pseudomonas* were detected during the elaboration process. The elaboration process was done in three replicates for each treatment.

Fruit samples were collected at different dates of the fermentation process: 1) beginning of fermentation process; 2) middle of fermentation process; and 3) final of fermentation process.

The analysis for the calcium content, firmness, phenol profile and antioxidant activity were performed.

Calcium analysis

Fifty fruits per replicate were washed three times in distilled water and dried using a filtering paper. The olive pulp was removed from the pit with a manual stoner. The seeds were also separated by the pit with a commercial hammer. The vegetal material was crushed with a thermobeater and 2 g was dried at 100 ºC for 24 hours and ashed in a muffle furnace at 550 ºC (1º C min^{-1}). The ashes were dissolved in 20 mL of 4% HNO_3. The calcium content was determined using an ICP-OES Perkin-Elmer 5300 DV spectrophotometer at 317.933 nm in a radial mode, with a concentric nebulizer and a cyclonic nebulization chamber. Results were expressed as mg kg^{-1} on dry weight (d.w.).

Sensory analysis of the fruits

Sensory analysis was performed by eight panellists trained according to the standardized norm of the International Olive Council [32]. The sensory properties of table olive, including abnormal fermentation, other defects, salty, bitterness, acidity hardness, fibrousness and crunchiness were assessed by the trained panel.

Firmness of the fruits

The method followed to determine the olive firmness was described by Morales-Sillero et al. (2020). Forty fruits per replicate were used for firmness determination using a TA-TX2 texture analysers (Stable Micro Systems, Godalming, UK) connected to a computer and fitted with a 30kg load cell. Each olive fruit was placed between a flat steel plate mounted on the machine. Olive firmness was measured by compression test, applying a force (N) to crush the olive through a 20 mm diameter probe to achieve a 6% deformation of the fruit diameter. The test speed and the trigger force were 0.5 mm·s^{-1} and 0.04903 N, respectively. Values were expressed as breaking force in Newton (N).

Determination of phenolic compounds

The phenolic profile analysis was carried out in 40 fruits per block with an Agilent 1100 model HPLC system (Hewlett-Packard, Waldbronn, Germany) following the method described by (Cabrera-Bañegil et al., 2017). 2g of homogenized samples was extracted in ultrasonic bath (P-Selecta ultrasonic bath, mod 516, Barcelona, Spain) with 10 mL of methanol, containing NaF 2 mM, during 30 min. After centrifuged (Thermo Scientific Sorvall Legend XT/XF centrifuge, with a F13-14x50c carbon fiber rotor, Thermo Fischer Scientific, USA) at 1677g at 4 ºC during

10 min, the extracts were filtered prior the injection into the HPLC system. Stock standard solutions of the phenols studied were accurately prepared and dissolved in methanol.

Statistical analysis

One-way ANOVA and Tukey's test were performed to determine significant differences between treatments. SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to perform ANOVA analysis. Outcomes were expressed as mean values and corresponded to the calcium treatments applied in each experiment. Statistical significance was accepted at the level of $p < 0.05$. Data were expressed as mean ± standard deviation.

Results

Calcium content

During table olives Spanish-style elaboration process, olives were spontaneously fermented in their corresponding fermentation tanks at the same chemical conditions. No lactic acid bacteria, enterobacteria, coliforms, Bacillus, and Pseudomonas were detected in the fermented olives for each calcium treatments (data not shown). Furthermore, the calcium content in the pulp, the pit, the seed and the whole fruit was monitored during the fermentation process. The pulp calcium content increased with the Ca-treatments (Figure 1a). A significant difference was found between tested concentrations. The increase of calcium content was 55.1% and 20.2% for Ca 1.0% and Ca 0.5% concentrations, respectively. The pit showed a higher calcium content than the pulp when Ca 0% was applied (Figure 1b). Nonetheless, this tendency changed when the calcium was used but without significant differences between calcium treatments within each sampling date. The content of calcium in the seed (Figure 1c) was significantly higher than in other tissues of the fruit (three times compared to the pulp and pit). Nevertheless, the contribution of the seed is not significant compared with the whole fruit due to its low weight and the seed high calcium content was not reflected on the total calcium content. The calcium content resulted significantly higher when the Calcium treatments were applied but no significant differences were found between the tested Ca concentrations. A statistical difference between Ca-treatments and Ca 0% was also registered in the whole fruit (Figure 1d). The calcium content of the fruit increased of the 13.4% and 36.5% compared to Ca 0%, with Ca 0.5% and Ca 1.0% concentrations, respectively.

Regarding the evolution of the Ca content during the elaboration process, no significant differences were found between sampling dates when the pulp, the pit, the seed or the entire fruit were considered. This result indicates that the calcium applied during the fruit growth development was not removed during the whole elaboration process.

Sensory analysis

A triangular test was carried out to verify if the tasters were able to differentiate not-treated Spanish-style table olives with those treated with pre-harvest calcium applications (Table 1). It can be said that the different samples were correctly identified in the different trials carried out. Thus, it could be statistically affirmed that tasters were able to differentiate between Ca-treated olives (0.5% and Ca 1%) and those without calcium addition. This is a very interesting result since tasters are able to discriminate samples based on their olfactory-gustative senses. However, it should be noted that the percentage of success for differentiation of the samples subjected to calcium treatments in the triangle test was less than the 66%. Results of the sensory analysis are shown in Table 2. Some significant differences were observed for each sensory attribute. The highest differences were found for bitter, hardness, and crunchiness. Values ranged from 2.1 to 3.5 for bitter and from 3.6 to 6.4 for hardness. Crunchiness also showed slight significant differences. For these sensory attributes, table olives subjected to calcium applications recorded the highest values. Nevertheless, the attributes related to negative sensations (i.e. salty, acidity, fibrousness, and color) did not show differences when Spanish-style table olives were compared to those subjected to pre-harvest calcium treatments.

Fruit firmness

The compression force (firmness) applied to the fruits significantly decreased during the elaboration process in Ca 0% and Ca 1.0%, while did not show variation in Ca 0.5% (Figure 2). Nevertheless, Ca-treatments increased the compression force in the fruit when compared with Ca 0% samples. On the first date of monitoring, a 22.8% and 43.8% increase of the compression force was registered for Ca 0.5% and Ca 1.0% concentrations, respectively. On the second date, the increase for Ca-treatments was 42.8% for Ca 0.5% and 68.5% for Ca 1.0%. For the third date, the tendency seen in the previous sampling dates changed: no significant differences were found between Ca concentrations recording an increase of 68.3% compared to Control.

The results showed that the compression force needed did not change significantly during the whole elaboration process for Ca 0.5% (Figure 2). However, the firmness decreased significantly at the second date for Ca 1.0% during the elaboration process. In addition, comparing the firmness of Ca-treatments on the second and third date, no significant difference on compression force were observed.

Phenol composition

During the elaboration process of table olives, the phenol composition was determined (Table 3). The most representative phenolic compounds were Hydroxytyrosol and Oleuropein, whereas phenols that showed the lowest concentration were Apigenin and Apigenin-7-O-glucoside. Phenol profile tended to decrease during the elaboration for some compounds, such as Hydroxytyrosol, Tyrosol, Oleuropein, PB1 and Verbascoside decreased, whereas the composition during the whole elaboration process did not change for others like Luteolin-7-O-glucoside, Luteolin, Epicatechin, p-coumaric acid, Quercetin-3-rutinoside, Apigenin and Apigenin-7-O-glucoside. This information was applicable to Ca 0% samples and also to the Ca-treated ones (Table 1).

The content of phenol composition decreased during the elaboration process. The results showed that there were significant differences between Control and Ca-treatments within each sampling date for all analysed phenols. The first date of monitoring showed that the concentrations of Hydroxytyrosol, Tyrosol and Verbascoside were included between 16.5% and 65.7%, higher for the Ca treated table olives compared to Ca 0% ones. Ca-treatments showed higher concentrations than Control samples for the second and third date (between 16.0% and 64.1% and between 23.6% and 80.8%, respectively). The highest contents for these phenolic components were found at the first sampling date for Ca 1.0% concentration: 331.4, 41.6 and 82.0 mg kg^{-1} for Hydroxytyrosol, Tyrosol and Verbascoside, respectively.

For Oleuropein and PB1, significant differences were found between the applied Ca concentrations and between Ca-treated fruits and Ca 0%. Thus, table olives submitted to Ca 0.5% and Ca 1.0% presented higher Oleuropein and PB1 concentrations than Control table olives (Table 3). At the first sampling date, the content of Oleuropein and PB1 was 29.8% and 12.7% higher than Ca 0% for Ca 0.5% treatment and 48.9% and 26.2% higher for Ca 1.0% treatment. At the second sampling date, the content of the same phenols was between 12.6% and 29.8% and between 26.1% and 49.0% higher than Ca 0% for Ca 0.5% and Ca 1.0% concentration, respectively. At the third sampling date the content was between 12.5% and 22.9% and between 26.3% and 37.7% higher than Ca 0% for 0.5 % and 1.0% Ca-treatments, respectively. The highest content of Oleuropein and PB1 was registered for Ca 1.0% treatment at the first sampling date (287.7 and 82.1 mg kg $^{-1}$, respectively).

The content of Luteolin-7-O-glucoside also decreased during the elaboration process. In particular, at the first sampling date, no significant differences were found between Ca 0% and the Ca 0.5%, whereas significant differences were seen for Ca 1.0% (the content was 31.1% higher than Ca 0%). For Ca 0.5% and Ca 1.0% treatments, the Luteolin-7-O-glucoside concentration at the second sampling date was 10.4% and 42.9% higher than Ca 0%, respectively. At the third sampling date, no significant differences were found between the Ca 0% and the Ca 0.5% treatment, whereas significant differences were seen for Ca 1.0% (the content was 43.0% higher compared to the Ca 0%). The highest concentration of Luteolin-7-O-glucoside was registered for Ca 1.0% treatment at the first sampling date (39.1 mg kg $^{-1}$).

The remaining analysed phenolic compounds did not show any significant differences between Ca 0% and the applied calcium treatments within each sampling date or between sampling dates within the same treatment. However, the highest contents were found at the first sampling date for Ca 1.0% concentration excepted for p-coumaric acid that showed the highest value for Ca 0.5% treatment. For some of the phenols, such as Hydroxytyrosol, Oleuropein and PB1 the results showed a significant difference between the phenolic content at different monitoring dates. The highest content appeared at the first date and decreased gradually. Tyrosol showed a significant difference with the first date and the second and third date, this last two did not present significant differences. The first date showed higher concentrations of Tyrosol. Verbascoside, did not show significant difference between the first date and the second one, but both dates showed a significant difference with the third one. This last monitoring date showed the lowest concentration out of the three. For the rest of the phenols mentioned before, not significant differences were recorded for the concentration between the different dates of monitoring.

Discussion

Table olives pre-harvest Ca-treatments implies an assimilation of this macronutrient in pulp, pit and seed. This absorption is beneficial in terms of firmness, bioactive compounds synthesis and antioxidant capability. Agreeing with other researchers, calcium is an important macronutrient that can be used at different stages of the elaboration process of table olives or in pre-harvest treatments in different cultivars. Previous studies (García-Serrano et al., 2020; Gouvinhas and Barros, 2021; Tsantili et al., 2008) reported pre-harvest foliar applications of an organic calcium compound to three different cultivars. The application in different moments of the fruit development enhanced the final Ca content, similarly to the data obtained in our research. Other studies indicated this enhancement of calcium content in the pulp of several fruits after Ca-treatments. For example, Manganaris et al. (2005) observed this increase for peach when Ca sprays were applied once a week during 10 or 6 weeks before harvest until commercial ripening of the fruit. In olive, Tsantili et al. (2008) reported an accumulation of calcium after spraying three times a $CaCl_2$ solution (0.65% w/v) throughout the month previous to harvest. More recently, (Morales-Sillero et al., 2021) demonstrated that foliar applications of Ca were also effective when applied during fruit development, at three different stages and suggested a potential positive influence along the Spanish-style elaboration process to be demonstrated with further analysis.

The results of our study showed a significant increase of calcium content in the pulp and the seed of the fruit when three Ca foliar applications were applied during the fruit development. Other studies also found an enrichment in calcium content in the seed of fruits after Ca-treatments. Ciccarese et al. (2013) observed an increase in berries when $CaCl_2$ was sprayed twice, from fruit set to veraison and from veraison to harvest. Our study showed that the calcium content on the seed did not change during the different stages of the elaboration process as table olives. Our results also highlighted a significant difference of Ca content between the seed and the other tissues of the fruit, showing values that were quite the double comparing the seed with the pulp. Ciccarese et al. (2013) concluded that the difference could be due to the translocation of the calcium from the skin to the seed, even though this was not confirmed, and further investigations are required. Harker & Ferguson (1988) explained that Ca-treatments are highly successful in the fruit, being this sink able to absorb Ca^{+2} better than the other portions of the tree with a later re-translocation of calcium. This study also mentioned than in mature fruits the absorption of calcium take place because of the cuticle cracking due to the growth of the fruit. According to this, cracking makes easier the absorption of Ca^{+2}, but to increase the fruit content, Ca-treatments should be applied.

Our study confirmed that the calcium content does not decrease through the elaboration process of table olives when Ca was applied to developing fruits on the tree (pre-harvest), so that no further applications were required in post-harvest. This positive effect can be attributed to the accumulation of calcium in the lamella region of the cell wall with the following stabilization (Tobias et al., 1992). Calcium is a phloem immobile nutrient; it only can be transported with normal water flow for its accumulation on the fruit. The cell wall permeability to water can be modify by calcium, so it can decide its own transport (Hocking et al., 2016). Calcium connects to the cell walls of the fruit once it is applied to the fruits in the field. During the elaboration process, the Ca included inside the fruit cell increases the rigidity of the membrane, strongly reducing the diffusion of calcium to the brine and immobilizing it through stable bonds, although this inhibitory effect depends on the pH of the brine solution during Spanish-style fermentation process (Brenes et al., 1994). In our study, the physico-chemical parameters have been artificially controlled to maintain all the experimental treatments under the same chemical conditions during the fermentation process. This was confirmed by our results: average Ca content in the pulp did not decrease during the elaboration process for table olives (Figure 1). Same trend was observed for the Ca content in the pit and in the seed. The high calcium content of the olives subjected to pre-harvest calcium treatments did not affect the microbial development and activity during the fermentation process, excluding a possible toxic effect of the Ca studied concentrations.

The sensory profile of the olives subjected to pre-harvest calcium applications showed appreciable differences between them (Table 1 and Table 2). It is known that the post-harvest calcium applied during Spanish-style table olives can contribute to a higher bitter taste in the final product (Martín-Vertedor et al., 2020; 2021). In fact, pre-harvest calcium application doses should not be excessively high as this could affect the sensory quality of the processed table olives. However, with the concentrations applied in this study, it can be concluded that the bitterness values were included in the normal range for this kind of olive processing protocol (Schaide et al., 2019). The application of Ca-treatments helped to maintain the fruit firmness even after the alkaline stage of the elaboration process, contributing to increase the quality of the olive by improving the fruit texture (Table 2; Figure 2). This aspect, together with the crunchiness, was also positively evaluated by the tasters in the olives subjected to pre-harvest calcium applications. Such result can provide an extra-quality value to this type of olives, indicating a potential market for pre-harvest Ca-treated Spanish-style table olives. Schaide et al. (2019) reported no bitterness and high acceptability of table olives elaborated with olive leaf extract according to the Spanish-style. The firmness of the fruit is also important to contrast disease attacks and prevent physiological disorders as reported by Rincón and Martínez (2015).

Calcium is the second most important mineral involved in the cell walls in plants and its main role is to maintain the structure of the wall (Jemrić et al., 2016). Tobias et al. (1992) indicated that the extra rigidity on the cell walls could be because the creation of calcium cross-linkage between pectic polymers. This study also mentioned that the calcium penetrating in the fruit was accumulated in the middle of the lamella region of the cell wall and then stabilized. This stabilization was caused by the formation of ionic bridges between pectic polysaccharides.

The effect of the Ca-treatments could be detected since the first date of sampling with the higher breaking force values registered for the Ca-treated fruits. Tsantili et al. (2008) also found an increase in firmness for table grapes after Ca-treatments were applied. Rincón & Martínez (2015) also addressed the importance of the quality preservation of fruits for the commercialization, characteristic that improved with the increased firmness after the application of Ca-treatments.

Despite the Ca content in the pulp did not decrease, during the elaboration process a rigidity decrease was observed. This could be because the elaboration process of Spanish-style uses lactic bacteria and other microorganism that can metabolise and break the vegetable fibres causing a decrease of the rigidity (Lanza, 2013). As said before, firmness decreased during the elaboration process for table olives. Thus, olives went through an alkaline treatment that modified the composition of the cell wall causing the olive softening. With the softening of the fruit, the quality of the olive decreases (García-Serrano et al., 2020). Therefore, there is a need of studying different strategies to avoid this process that cause a loss of rigidity in olives for ensure high quality products.

The application of pre-harvest Ca-treatments also increased the content of some phenolic compounds during Spanish-style elaboration process (Table 3). The highest content was achieved with the highest Ca concentration. The obtained results are consistent with previous studies where an increase of phenolic compounds was registered for sweet cherry fruits when trees were sprayed with calcium solution once a week from flowering until two weeks before harvest (Vangdal et al., 2008). As said before, Ca-treatments increased the concentration of calcium in the olives stimulating the synthesis of the phenolic compounds (Miceli et al., 1999), being this the possible explanation for our results. Charoenprasert & Mitchell (2014) reported that several factors may have a huge effect on the increment of phenolic compounds in olive fruits, existing differences between olive cultivars. The Ca content in the fruit can directly contribute to the phenol concentration along the elaboration process. Since this macronutrient gives rigidity and permeability to the sample, it is likely that phenols, that are functional water-soluble compounds, do not diffuse as easily to the brine when calcium is present in high concentration in the matrix. The increase in firmness due to an industrial scale application of $CaCl_2$ can lead to an increase on hydrophilic molecules concentration in black olives, as reported by Martín-Vertedor et al. (2021). This was seen also for green olives but with less intensity (Martín-Vertedor et al., 2020). Both reported studies underlined the importance of controlling the addiction of $CaCl_2$ to regulate the increase of acrylamide and protect consumer's health.

As said before, the increase in phenol compounds in Ca-treated olives was higher even after fermentation in brine, resulting in a healthier functional food for the consumer. In our study, Hydroxytyrosol and Tyrosol were found as the main phenolic compounds. Hydroxytyrosol has a wide range of health benefits, such as, cardioprotective, anticancer, neuroprotective, antimicrobial, and other effects. Tyrosol is an effective cellular antioxidant but is also effective against hypertension, atherosclerosis, coronary heart disease, chronic heart failure, insulin resistance and obesity (Marković et al., 2019). According to these findings, the consumption of Ca-treated table olives should result more beneficial than not-treated ones.

Moreover, the content of phenolic compounds increased with increasing Ca applied concentration treatments and consequently the antioxidant activity, being the factors highly correlated. Other studies also confirmed this correlation Giménez et al. (2014) in sweet cherries after the application of acetylsalicylic acid. Ozturk et al. (2014) indicated the increasing of antioxidant activity in plum fruits by applying calcium treatments. These authors indicated that this fact contributes to provided anti-mutagenic and anti-carcinogenic benefits for humans. Other researchers (Martínez-Esplá et al., 2014) related the increase of antioxidant activity with the activity of certain enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and polyphenol oxidase. When these enzymes and the phenols are together, they are able to collect free radicals and reactive oxygen species that provoke the increasing of the antioxidant activity. The increase in antioxidant activity caused by the increase in phenol profile due to pre-harvest Ca-treated provides a final product with a health benefit for human. This fact also provides the possibility of having a final product on the market with longer shelf-life or by reducing the degradation process of the table olives, especially when they are marketed with some quality mark, such as organic farming.

Conclusion

The application of Ca-treatments during the fruit development (pre-harvest) by spraying 0.5% and 1.0% $CaCl_2$ concentrations enhanced the quality of the Spanish-style table olives by increasing the Ca content on the fruit, the firmness and the phenolic content. The fermentation process of the olives subjected to pre-harvest calcium treatments occurred regularly, without registering toxic effects of the calcium (no inhibition of the microorganisms responsible of the fermentation process). Further and deep studies about the effect of pre-harvest calcium treatments on the inhibition of spoilage bacteria are required.

An important result highlighted in our study was that pre-harvest Ca-treatments provided a better firmness to the olives throughout the fermentation process. This aspect is particularly important for those varieties, as 'Ascolana tenera', that are characterized by a soft pulp and are particularly sensible to damages during harvest or post-harvest manipulation. An increased pulp firmness could allow the mechanization of the fruit handling during harvest and post-harvest. Moreover, the effect of additional post-harvest Ca applications should be studied to verify if an improvement of the organoleptic characteristics (hardness and crunchiness) of the fruit is possible in order to give a better sensory experience to the consumers. Our results also demonstrated that fruit quality parameters can be increased by Ca-treatments during the fruit development. In fact, the accumulation of phenolic compounds was stimulated and their content did not decrease during the elaboration process. This result has an important consequence resulting the table olives with higher phenols and antioxidant activity an outstanding functional food for the consumers.

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