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5. KAOLIN, *ASCOPHYLLUM NODOSUM* AND SALICYLIC ACID MITIGATE EFFECTS OF SUMMER STRESS IMPROVING HAZELNUT QUALITY

5.1. Abstract

Background: Difference strategies are needed to mitigate the negative impact or to increment fruit quality. The effect of spraying kaolin (K), *Ascophyllum nodosum* (An) and salicylic acid (SA), in trees with and without irrigation, on quality and sensorial attributes of hazelnut (Grada de Viseu cultivar) was investigated during two consecutive years (2016 and 2017) in a commercial orchard located in Moimenta da Beira, Portugal.

Results: The treatments affected positively the biometric parameters, nut and kernel, weight, length, width, thickness and volume as well as the vitamin E level, antioxidant activity and some individual phenolic content, such as protocatechuic acid, gallocatechin, catechin and epicatechin. The levels of amino acids in hazelnut kernels decreased in all the assayed treatments, while the kernel colour and sensorial attributes were not affected by the treatments. Hazelnut physical properties (nut and kernels), chemical and phytochemical composition and antioxidant activities were positively related.

Conclusion: The application of K, An and SA improve the hazelnut tree response to the climate change, without compromising the hazelnut chemical and sensorial quality. Furthermore, due to the similar observations on the same treatments with and without irrigation, it can be stated that K, An and SA can be effective and cost-effective tools to mitigate summer stress in rainfed orchards.

Keywords: *Corylus avellana*; climate change; foliar sprays; nut quality; phytochemicals; antioxidant activity.

5.2. Introduction

European hazelnut is one of the most important nut crops in terms of worldwide production. In 2017, 1 006 178 t of hazelnuts were produced in the world, being Turkey the largest producer, responsible for 70% of hazelnut world production. In Portugal, the hazelnut production orchards are mainly located in North, due to its favourable edaphoclimatic conditions (Martins et al., 2015). Nonetheless, the irregular pattern of climate conditions in the recent years, has contributed to obtain irregular yields with reflex in chemical and physical properties, and, thus, in the nut nutritional quality. Hazelnut, due to its sensory pleasant characteristics, constitutes one of the most important raw material for the pastry and chocolate industry (Fallico et al., 2003), being highly considered since it adds flavor and texture to bakery, confectionery, cereal, dairy, salad, entrée, sauce and dessert formulations. In addition, to provide a desirable flavour to different food, it also has an important role in human nutrition due to its protein, oil, vitamin E and mineral contents. Hazelnuts are a rich source of monounsaturated and polyunsaturated fatty acids and vitamin E (Ozdemir and Akinci, 2004). Likewise, other fruit crops, hazelnut must face new challenges as result of the recent changes in climatic conditions (Kröner et al., 2016). Excessively high temperatures in summer and irregular precipitation distribution along the year will impact several physiological and metabolic processes (Cabo et al., 2019), with negative implications in plant growth, productivity and nut quality. Recent studies have showed a positive effect against heat and water stress of foliar sprayers containing kaolin (K) a chemically inert white mineral with excellent reflective properties (Brillante et al., 2016), salicylic acid (SA) and *Ascophyllum nodosum* (An) a natural seaweed based product in grapevine (Brillante et al., 2016), olive (Brito et al., 2018; 2019); sweet cherry (Correia et al., 2019) and hazelnut (Cabo et al., 2019). Although a recent study showed a positive effect of K and An in physiological performance of hazelnut trees during summer stress (Cabo et al., 2019), no information is available about K, An and SA effect on hazelnut fruit composition and nutritional and sensory quality. Thus, a study was set to evaluate the impact of leaf spraying with K, An and SA on 'Grada de Viseu' cultivar hazelnut composition and sensorial attributes. 'Grada de Viseu' is one of the most popular commercial cultivar produced in Portugal, and it is also known as 'Barcelona' in the USA, 'Castanyera' in Spain, 'Fertile de Coutard' in France (Mehlenbacher and Miller, 1989; Boccacci et al., 2013), preferencially used in in-shell market. For the in-shell market segment, the relative size and appearance of the nuts are the most important characteristics, with a

preference for large, shiny nuts (Baldwin, 2015). Due to their very special nutritional value and high content of vitamin E and some antioxidant compounds, hazelnuts are a major role in human nutrition (Köksal et al., 2006).

The results from this study, will allow understanding if the application of such substances can be used to reduce the negative impact of climate change conditions, and if they may have any negative impact in hazelnut fruits overall quality attributes.

5.3. Material and methods

5.3.1. Experimental design and sampling

Experiment was conducted during two consecutive years (2016 and 2017) on a hazelnut rainfed commercial orchard with adult trees in full production ('Grada de Viseu' cultivar), planted at 4 x 5 m, located in Moimenta da Beira, Viseu, North of Portugal (40°59'N, 7°37'W, altitude 644 m). The climate in this region is Mediterranean, characterized by warm-temperatures, with dry and hot summers, according to the Köppen-Geiger climate classification, the climate is Csb (Kottek et al., 2006). The monthly average air temperature and precipitation recorded in the orchard by an automatic weather station (IMT280, iMETOS, Weiz, Austria), during the experimental period are shown in Table 5.1.

The experiment comprised eight treatments: Control (C), in which trees were sprayed with distilled water; irrigation (I); kaolin (K), trees sprayed with 4 % (prepared in water; 4 g/100 mL of water) K particle film (Surround WP; Engelhard Corp., Iselin, NJ); *Ascophyllum nodosum* (An), trees sprayed with 0.15 % (v/v) An is an seaweed-based extract (SPRINTEX NEW® L.); SA (salicylic acid), trees sprayed with 0.01 % SA (v/v); K with irrigation (Ki), An with irrigation (Ani), SA with irrigation (SAi). The irrigation regime adopted was calculated on the basis of crop potential evapotranspiration [(ETc = ETo × Kc × Kr), where ETo is the reference evapotranspiration for the previous day calculated using the Penman–Montheith equation) (Allen et al., 1998), Kc is the crop coefficient considered on the basis of the crop features and Kr is the ground cover reduction coefficient]. Water was supplied using a drip irrigation system. On average, trees were irrigated 1.5 hr, 3 days a week in July and 1 hr 2 days a week in August, with a total of 300–400 L/year. The field experiment was arranged as a factorial design, with five homogeneous trees per treatment, 40 trees in total. Throughout the

experiment, standard cultural practices (pruning, disease and pest control) were regularly implemented following the local practices.

The treatments were applied, in the absence of wind, on 23rd June 2016 and 21st June 2017. The irrigation season in both of years, started at the beginning of July and ended at the end of August, corresponding to the nut and kernel development (Martins et al., 2015; Cabo et al., 2019). Fruits from each tree were harvested simultaneously at the commercial maturity stage on 29th September 2016 and 22nd September 2017.

Table 5.1. Weather conditions in 2016 and 2017, in Moimenta da Beira, Viseu, Portugal.

Parameter		Month											
		January	February	March	April	May	June	July	August	September	October	November	December
Air Temperature (ºC) *	2016	8.1	6.8	7.5	10.1	13.3	18.3	22.9	22.8	18.9	14.0	8.5	5.5
	2017	5.2	8.5	10.0	13.5	16.3	21.0	21.5	21.4	17.8	17.3	9.4	6.6
Precipitation (mm) **	2016	232.9	115.8	51.8	175.0	145.0	5.6	5.1	3.3	18.5	58.9	116.1	43.4
	2017	26.4	110.0	39.4	8.6	64.3	3.8	3.3	12.2	13.0	17.8	39.9	71.1

* average temperature of each month
** total precipitation of each month

5.3.2. Yield and biometric properties of hazelnut fruits

At harvest, in each treatment yield (kg/ tree) was determined as well as the number of nuts per kilogram to estimate the number of fruits per tree. For each treatment, a sample of 30 hazelnuts were randomly chosen. Nut and kernel length, width, and thickness were measured using a digital calliper (0.01 mm sensitivity). The hazelnut and kernel weights were measured with a precision balance (0.1 g sensitivity) and the volume was determined using the liquid displacement method. The percentage of kernel (kernel %) was calculated as (kernel weight/ nut weight) x 100, the nut roundness index (RI) and kernel roundness index (kernel RI), was calculated as nut length/ nut width and kernel length/ kernel width, respectively. Nut and kernel colour were measured at opposite sides with a chroma meter (CR-300, Minolta, Japan) that expresses colour as L, a^* and b^* values. Chroma (C^*) an effective parameter for describing visual colour appearance was calculated according to $C^* = (a^{*2} + b^{*2})^{½}$ (Ozdemir and Akinci, 2004)

5.3.3. Chemical and phytochemical composition

5.3.3.1. Amino acids

The extraction and purification of free α-amino acids was performed according to Gomes and Rosa (Gomes and Rosa, 2000) procedure, with some modifications. For extraction, 5 mL of 70% aqueous methanol was added to 200 mg fresh weight (fw) of each hazelnut sample (kernels were blended in to a fine powder prior to analysis) and heated at 70 ºC for 10 minutes and then centrifuged at 4000 rpm for 20 minutes (Centric 250, Uniequip, GmbH, Fraunhoferstraße, Germany). The supernatants were collected, and the final volume was adjusted with 70% aqueous methanol to 10 mL. Then, aliquots of 2 mL were evaporated and resuspended in 2 mL of 0.1 M HCl. Mini-columns of 1 mL (Chromabond from Macherey-Nagel) were connected to a solid phase extraction vacuum system (Gilson) were used, and eluted with 0.5 mL of 0.1 M HCl before filled up to 2 cm with a cation exchange resin, Dowex (H^+) 50WX8-499 (Sigma-Aldrich Chemicals, St Louis, MO, USA). Simultaneously, amino acids standards were loaded onto the columns and washed with 5 mL of 0.1 M HCl. Free α-amino acids were eluted with 4 x 2.5 mL of 7 M NH_3 (Merck, Darmstadt, Germany). After evaporation, the residue was resuspended in 0.3 mL of distilled water, filtered [(PTFE 0.2 µm,

Ø 13 mm (Teknokroma, Spain)] and kept in vials at -20 ºC until further analysis. Amino acids were determined by high performance liquid chromatography-diode array detector (HPLC-DAD) after a pre-column derivatisation with *O*- phthalaldehyde/2-mercaptoethanol. The HPLC-DAD system was equipped with a C18 column (150 x 46 x 5 µm) (ACE® HPLC columns, Advanced Chromatography Technologies, Ltd., Aberdeen, Scotland) with a mobile phase of two solvents: solvent A, with 0.1 M sodium acetate pH 6.95 + absolute methanol and tetrahydrofurane (TFA) (92.5:5.0:2.5) and solvent B, composed by absolute methanol + TFA (97.5:2.5). The gradient was binary starting at 0 min with 0% of solvent B, 6 min with 10% solvent B, 8 min 15% solvent B, 12 min 25%, 18 min 40%, with a flow of 1.2 mL min^{-1}, in a run length of 18 min. The injection volume was 10 µL. The identification and quantification of amino acids were performed with response factor method and external standards after adjustment through regression lines. The results were expressed as mg g^{-1} fw (fresh weight).

5.3.3.2. Vitamin E (α-Tocopherol) levels

The levels of α-tocopherol in the hazelnut samples were determined by HPLC-DAD system, according to the method of Gueguen et al. (Gueguen et al., 2002) with several modifications. Ten mL of a solvent mixture methanol:dichloromethane (1:1) was added to 1 g of fw hazelnut kernels, mixed thoroughly in a vortex and sonicated for 20 min. Then, mixtures were centrifuged at 4000 rpm and room temperature for 10 minutes (Centric 250, Uniequip, GmbH, Fraunhoferstraße, Germany). The supernatants were collected and filtered (PTFE 0.2 µm, Ø 13 mm; Teknokroma, Spain) to HPLC amber vials (Chromabond 2-SVW(A) ST-CPK, Sigma-Aldrich, Tauferkichen, Germany) and immediately injected in HPLC-DAD system to avoid any substantial losses of vitamin E by light degradation. The HPLC system was set with a C18 column (150 x 46 x 5 µm) (ACE® HPLC columns, Advanced Chromatography Technologies, Ltd., Aberdeen, Scotland), an eluent of absolute methanol with 0.1% of trifluoroacetic acid (TFA), in an isocratic gradient with a run length of 15 minutes, flow of 1 mL min^{-1} and injection volume 20 µL. The detection of vitamin E was performed at 296 nm wavelength, using the retention time (7.06 min.) and the UV spectra of the peaks obtained in the chromatograms by comparing with external commercial standard of α-tocopherol (Sigma-Aldrich, Tauferkichen, Germany). A calibration curve of commercial standard α-tocopherol at

different concentrations was injected in HPLC system and results were used to express the content of vitamin E in hazelnut sample as mg α-tocopherol g^{-1} fw.

5.3.3.3. Total sugars and starch

Total sugars and starch of hazelnuts were determined according to the method of Ni et al. (2009). Forty mg of fw of each hazelnut sample was mixed with 2 mL of pure acetone and sonicated for 5 min. in order to remove interfering pigments. Then, the extracts were centrifuged at 11000 rpm, 15 min and 4 °C (Eppendorf Centrifuge 5804 R, Hamburg, Germany). The supernatants were rejected and the residues (pellets) used to extract total sugars and starch. For total sugars extraction, 2.5 mL of 80% aqueous ethanol was added to each residue, the mixtures were placed in a thermoblock at 80 ºC for 30 min. and centrifuged at 11000 rpm, 15 min. and 4 °C (Eppendorf Centrifuge 5804 R, Hamburg, Germany). The supernatants were used to quantify total sugars and the residues were used to extract starch by adding 2 mL of 1.1 % HCl at 100ºC for 30 min. The mixtures were cooled in ice, centrifuged at 11000 rpm, 15 min. and 4 °C (Eppendorf Centrifuge 5804 R, Hamburg, Germany) and supernatants collected. All supernatants were reserved at -20 ºC until total sugars and starch determination by colorimetry. The total sugars and starch quantification were performed by anthrone method by adding 500 µL of anthrone reagent (0.2% of anthrone in concentrated H_2SO_4) to 100 µL of each hazelnut extract prepared previously and placing the mixtures at 100 ºC for 10 minutes. After cooling in ice, the absorbance values were recorded at 630 nm against blank (H_2O instead of sample). The results were expressed as mg of glucose equivalent g^{-1} fw and mg of starch equivalent g^{-1} fw using the corresponding calibration curves.

5.3.3.4. Total phenolic content (TPC)

TPC was determined using a classical colorimetry method performed in 96-microplate wells (Costar 3590, Corning, NY, USA) adapted from Singleton and Rossi (Singleton and Rossi, 1965). Forty mg of fw sample was added to 1 mL of 70% aqueous methanol, heated at 70 ºC for 30 min. The mixtures were then centrifuged (Eppendorf Centrifuge 5804 R, Hamburg, Germany) at 11000 rpm, 15 min. and 4 °C. The supernatants were collected and filtered with Spartan filters (0.2 µm) to amber vials (Chromabond 2-SVW(A) ST-CPK, Sigma-Aldrich,

Tauferkichen, Germany) and preserved at -20ºC until further analysis of total phenolics, total flavonoids *ortho*-diphenols and antiradical/antioxidant capacities. For TPC, 20 µL of each extract was added to each microplate well, followed by addition of 100 µL of Folin-Ciocalteu´s phenol reagent (1:10 in dd H_2O) and 80 µL of 7.5% Na_2CO_3. The microplates were then heated at 45 ºC for 15 min. in dark, and the absorbance values were recorded at 765 nm wavelength in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) against blank (all reagents and 70% aqueous methanol instead of extract). The results were then expressed as mg gallic acid equivalent (GAE) g^{-1} fw, using a calibration curve of gallic acid at different concentrations.

5.3.3.5 Total flavonoid content (TFC)

TFC was performed using the same extract prepared for TPC and quantified by classical colorimetry method (Dewanto et al., 2002) in 96-microplate wells (Costar 3590, Corning, NY, USA) adopting the following procedure. Twenty-five µL of each extract was added to each microplate well followed by addition of 100 µL of ultra-pure water and 10 µL of 5% $NaNO_2$. The microplates were then incubated at room temperature for 5 min. in a dark environment. Then, 15 µL of 10% $AlCl_3$ was added to each well and the microplates were once again incubated at room temperature in a dark environment but for 6 min. Fifty µL of NaOH 1 M followed by 50 µL of ultra-pure water were then added and mixed thoroughly and the absorbance values were then recorded at 510 nm in microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) against blank (all reagents and 70% aqueous methanol instead of extract). The results were expressed as mg catechin equivalent (CAE) g^{-1} fw using a calibration curve with commercial standard of catechin at different concentrations.

5.3.3.6. Ortho-diphenols (o-DP)

The content of *o*-DP in hazelnut samples was determined according the method of Garcia et al. (2012) but conducted in a 96-microplate wells (Costar 3590, Corning, NY, USA). Twenty µl of hazelnut extract, prepared previously, was added to each microplate well and

mixed with 100 µL of ultra-pure water, 80 µL of phosphate buffer (pH 6.5; 0.1 M) and 160 µL of 5% molibdate ($Na_2MoO_42H_2O$) solution (Sigma-Aldrich, Tauferkichen-Germany). The microplates were then placed in dark at room temperature for 15 min. and the absorbance values were recorded at 350 nm against blank (all reagents and 70% aqueous methanol instead of extract) in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results were expressed as mg caffeic acid equivalent (CAFE) g^{-1} fw using a calibration curve with commercial standard of caffeic acid at different concentrations.

5.3.3.7. Individual phenolic composition

Both phenolic profile and content were determined by HPLC-DAD (Aires et al., 2013) system equipped with a C18 column (250 x 46 mm, 5 mm, ACE HPLC Columns, Advanced Chromatography Technologies Ltd, Abeerden, Scotland, UK), elluents composed by water with 0.1% of TFA as solvent A, acetonitrile with 0.1% TFA as solvent B in a gradient of: 0% solvent B at 5 min., 20% solvent B at 15 min., 50% solvent B at 30 min., 100% solvent B at 50 min., 0% solvent B at 60 min. The flow rate and injection volume used was 1 mL min^{-1} and 10 µL, respectively. The chromatograms were recorded at 254, 280, 320 and 370 nm. The peak retention time, UV spectra, UV max absorbance bands and by comparison with external commercial standards (Extrasynthese, Cedex, France) were used to identify each polyphenol detected. Prior to HPLC determination extracts were prepared by a hot (70 ºC for 30 min) solid: liquid extraction (49 mg fw^{-1} of hazelnut mixed with 950 µL 70% aqueous methanol and 50 µL of internal standard (the phenolic naringin). The extracts were then centrifuged (Eppendorf Centrifuge 5804 R, Hamburg, Germany) at 11000 rpm, 15 min. and 4 °C, and the supernatants collected and filtered with Spartan filters (0.2 µm) to amber vials (Chromabond 2-SVW(A) ST-CPK, Sigma-Aldrich, Tauferkichen, Germany). The samples were stored at -20ºC until HPLC analysis. The content of each phenolic was calculated using the internal standard method. The results were expressed as mg g^{-1} fw.

5.3.4. Antiradical and antioxidant activity

5.3.4.1. DPPH radical scavenging activity

The DPPH scavenging activity of hazelnut extracts was determined by colorimetry (Siddhraju and Becker, 2003) in a 96-microplate wells. Briefly, 285 µL of freshly DPPH radical solution (Sigma-Aldrich, Tauferkichen-Germany) (4 mg DPPH in 100 mL of ethanol 95%) was added to 15 µL of each extract previously prepared, followed by incubation at dark room temperature for 30 min. Then, the absorbance values were recorded at 517 nm in a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). A blank (all reagents and extraction solvent instead of sample) was considered. The results were expressed as % DPPH radical scavenging activity using the next formula: % DPPH scavenging capacity = (Absorbance blank – Absorbance sample extract / Absorbance blank) x 100.

5.3.4.2. Ability to reduce Fe^{3+} into Fe^{2+} (FRAP)

FRAP activity was determined by a colorimetry method (Benzie and Strain, 1996) conducted in 96-microplate wells. A fresh daily FRAP reagent solution was prepared: 300 mM, pH 3.6, of sodium acetate buffer with 10 mM 2,3,5-Triphenyltetrazolium chloride (TPTZ) solution (Sigma-Aldrich, Tauferkichen-Germany) (40 mM HCl as solvent) and 20 mM iron (III) chloride solution (Sigma-Aldrich, Tauferkichen-Germany) in a volume ratio of 10:1:1, respectively. Then, 25 µL of each extract was added to each microplate well and also 275 µL of FRAP reagent solution. The microplates were placed in a dark room temperature for 5 min and then absorbance values were recorded at 593 nm (MultiskanTM FC Microplate Photometer, USA) and $FeSO_4$ (Sigma-Aldrich, Tauferkichen, Germany) was used as control standard and the results were expressed as µmol $FeSO_4$ equivalents g^{-1} fw.

5.3.4.3. Inhibition of lipid peroxidation (ILp)

ILP was determined according to Ruberto et al. (2000) using an egg yolk homogenate as lipid rich media. To each microplate well, 20 µL of egg yolk (10% of egg yolk in 0,1 M phosphate buffer, pH 7.4) was added followed by 5 µL of $FeSO_4$ (1mM) (Sigma-Aldrich,

Tauferkichen-Germany), 20 µL of extract and 65 µL of ultrapure water. Microplates were then incubated at 37 ºC in a dark environment for 15 min. After that, 50 µL of 50% aqueous trichloroacetic acid (TCA) (Sigma-Aldrich, Tauferkichen-Germany) and 100 µL of thiobarbituric acid (TBA) (Sigma-Aldrich, Tauferkichen-Germany) (0.8% in phosphate buffer) were added and microplates incubated at 95ºC for 15 min. The absorbance values were recorded at 532 nm (MultiskanTM FC Microplate Photometer, USA). For the control, a complete oxidized extract (egg yolk + $FeSO_4$, without extract), the same procedure was adopted. The results were expressed as percentage of inhibition lipid peroxidation, calculated as: Inhibition (%) = $[1-(A_{sample}/A_{control})] \times 100]$.

5.3.5. Sensorial analysis

Hazelnuts from each treatment were evaluated by a panel of twelve participants aged between 35 and 50 years old, trained and experienced in sensorial trials. Panellists were non-smokers and refrained from wearing perfume and drinking or consuming foods that could affect performance, one hour before tasting. Tastings took place in a controlled environment room and with all the material needed for each task, according to the norm described in the International Organization for Standardization ISO standard 8589:2014 (Sensory, 2019).

Tree hazelnut fruits were presented to each panellist for each treatment. Samples were presented randomly, coded with a three-digit code number in white Pyrex plates. Mineral water and toasts were given between the evaluations to restore the initial tasting conditions. The sensory characteristics of the hazelnut samples were assessed using the quantitative descriptive analysis profiling method, following the procedure described in the (ISO) standard 13299:2016 (Sensory, 2019). A list of fourteen hazelnut attributes, adapted from Donno et al. (2013) and a structured scale from 1 (absence of the characteristics) to 5 (maximum intensity) points was used.

5.3.6. Statistical analysis

Statistical analysis was performed using the Software SPSS V.25 (SPSS IBM, Orchard Road-Armonk, New York, NY). Statistical differences were evaluated by one-way and two-way analysis of variance (ANOVA), followed by Tukey´s multiple range test ($P<0.05$) for

chemical data and, Duncan´s test ($P<0.05$) for sensory data. Pearson's correlation coefficients between antioxidant activities and phytochemical levels were calculated in order to evaluate the importance of phytochemicals in antioxidant activities of hazelnut kernels and how this is related with foliar sprayer's treatments.

5.4. Results

5.4.1. Yield and biometric properties of hazelnut fruits

The results for hazelnut yield and biometric properties are presented in Tables 5.2 and 5.3, respectively. In general, statistically significant differences were found between treatments for hazelnuts (Table 5.2) and kernels (Table 5.3), as well as for their respective colour attributes (Figures 5.1. and 5.2.). Similar tendency was observed for both nuts and kernels in which fruits treated with K and SA showed higher mass, length, width, thickness, and volume compared to the control fruits , with or without irrigation (Tables 5.2. and 5.3.). The number of fruits per kg in these treatments were lower, meaning that under these exogenous compounds the fruits reached higher size compared to control. Nonetheless, regarding colour attributes of nuts (Figure 5.1.) and kernels (Figure 5.2.), year and treatments affected in a different way the average values of L^*, a^*, b^*, and C^*, not showing any consistent trend between treatments and year, suggesting that treatments had not interference in the hazelnut (shells and kernels) colour properties.

Table 5.2. Biometric parameters of nuts (30 fruits per treatment), with different treatments, collected in two consecutive years (2016 and 2017) from an orchard in Moimenta da Beira Region, north of Portugal.[1-3]

Year	Treatment	*Yield (kg/tree)*	*Number of fruit per kilogram*	*Weight (g)*	*Length (mm)*	*Width (mm)*	*Thickness (mm)*	*Roundness Index (RI)*	*Volume (cm^3)*
2016	C	4.61 ± 0.64 a	364 ± 67 b	2.83 ± 0.50 a	19.68 ± 1.39 ab	20.16 ± 1.19 a	17.65 ± 1.17 a	0.98 ± 0.04	3.34 ± 0.76 a
	I	5.70 ± 3.26 a	299 ± 62 a	3.47 ± 0.68 bc	19.97 ± 1.17 abc	21.15 ± 1.27 bc	18.78 ± 1.54 b	0.95 ± 0.05	3.84 ± 0.90 bc
	K	3.62 ± 0.68 a	297 ± 41 a	3.43 ± 0.47 bc	20.76 ± 1.20 cd	21.77 ± 1.18 cd	18.92 ± 1.14 b	0.95 ± 0.04	3.86 ± 0.62 bc
	Ki	6.00 ± 2.14 a	271 ± 34 a	3.75 ± 0.46 c	20.92 ± 1.30 d	22.03 ± 0.93 d	20.10 ± 1.11 c	0.95 ± 0.06	4.34 ± 0.66 d
	An	4.87 ± 0.61 a	370 ± 63 b	2.78 ± 0.47 a	19.35 ± 1.32 a	19.65 ± 1.63 a	17.52 ± 1.17 a	0.99 ± 0.06	3.66 ± 0.80 ab
	Ani	7.68 ± 3.14 a	275 ± 31 a	3.68 ± 0.41 c	20.66 ± 1.27 cd	21.68 ± 1.15 bcd	18.73 ± 0.75 b	0.95 ± 0.05	4.18 ± 0.64 cd
	SA	3.54 ± 1.68 a	307 ± 53 a	3.35 ± 0.56 b	19.99 ± 1.52 abc	20.92 ± 1.45 b	18.54 ± 1.28 b	0.96 ± 0.05	3.88 ± 0.78 bc
	SAi	5.49 ± 1.32 a	276 ± 34 a	3.68 ± 0.51 c	20.40 ± 1.19 bcd	21.34 ± 1.16 bcd	18.73 ± 0.88 b	0.96 ± 0.05	4.10 ± 0.76 bcd
	P	n.s.	***	***	***	***	***	n.s.	***
2017	C	2.77 ± 0.57 b	388 ± 54 d	2.62 ± 0.32 a	18.84 ± 1.02 a	19.81 ± 1.19 a	17.31 ± 1.18 a	0.95 ± 0.04 a	2.70 ± 0.54 a
	I	2.85 ± 0.88 a	308 ± 32 ab	3.29 ± 0.34 de	19.66 ± 0.71 b	21.03 ± 1.04 c	18.31 ± 0.73 c	0.94 ± 0.05 a	3.80 ± 0.41 cd
	K	2.77 ± 0.89 a	318 ± 39 ab	3.19 ± 0.37 cd	20.08 ± 1.06 bc	20.88 ± 1.04 c	18.20 ± 0.86 bc	0.96 ± 0.05 ab	3.48 ± 0.59 bc
	Ki	2.79 ± 0.70 a	297 ± 26 a	3.40 ± 0.30 de	20.54 ± 0.74 d	21.25 ± 0.83 c	18.60 ± 0.78 c	0.97 ± 0.04 ab	3.73 ± 0.51 cd
	An	3.28 ± 1.19 b	357 ± 48 cd	2.85 ± 0.39 b	19.59 ± 1.02 b	20.67 ± 1.00 bc	17.66 ± 1.17 ab	0.95 ± 0.04 a	3.32 ± 0.48 b
	Ani	3.18 ± 1.66 a	296 ± 40 a	3.43 ± 0.48 e	20.67 ± 1.20 c	20.95 ± 0.96 c	18.22 ± 0.93 bc	0.99 ± 0.06 b	3.80 ± 0.65 cd
	SA	2.89 ± 1.17 a	288 ± 31 a	3.51 ± 0.36 e	20.52 ± 1.12 c	21.24 ± 0.88 c	18.60 ± 1.12 c	0.97 ± 0.06 ab	3.88 ± 0.42 d
	SAi	1.88 ± 0.59 b	336 ± 44 bc	3.03 ± 0.43 bc	19.56 ± 0.96 b	20.24 ± 1.00 ab	18.01 ± 0.89 bc	0.97 ± 0.04 ab	3.50 ± 0.51 bc
	P	n.s.	*	***	***	***	***	*	***
	Year (Y)	***	**	***	*	**	***	n.s.	***
	Treatment (T)	n.s.	***	***	***	***	***	n.s.	***
	Y x T	n.s.	***	**	*	***	**	*	n.s.

[1]Values expressed as mean ± standard deviation (SD). Abbreviation means: (C)-Control; (I)-Irrigation; (K)-Kaolin; (Ki)-Kaolin with irrigation; (An)-*Ascophyllum nodosum*; (Ani)-*Ascophyllum nodosum* with irrigation; (SA)-Salicylic Acid and (SAi)-Salicylic Acid with irrigation.
[2]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at P<0.05 by Tukey test.
[3]The symbols means: n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001.

Table 5.3. Biometric parameters of kernels with different treatments (30 fruits per treatment), collected in two consecutive years (2016 and 2017) from an orchard in Moimenta da Beira Region, north of Portugal.[1-3]

Year	Treatment	*Kernel %*	*Weight (g)*	*Length (mm)*	*Width (mm)*	*Thickness (mm)*	*Kernel Roundness Index (RI)*	*Volume (cm^3)*
2016	C	39.57 ± 0.25 a	1.12 ± 0.25 a	14.16 ± 1.13 a	12.94 ± 1.67 a	10.95 ± 1.67 a	1.11 ± 0.16 c	1.36 ± 0.42 a
	I	43.51 ± 2.48 cd	1.51 ± 0.32 bc	14.83 ± 0.87 bcd	14.87 ± 1.57 cd	12.86 ± 1.53 cd	1.01 ± 0.09 ab	1.78 ± 0.48 bc
	K	41.55 ± 4.28 abcd	1.42 ± 0.23 b	15.29 ± 0.95 dc	14.47 ± 1.61 bc	12.38 ± 1.29 bc	1.07 ± 0.12 bc	1.64 ± 0.34 b
	Ki	42.24 ± 2.49 abcd	1.59 ± 0.24 c	15.14 ± 1.00 cde	15.01 ± 1.18 cd	13.43 ± 1.21 de	1.02 ± 0.11 ab	1.92 ± 0.37 c
	An	40.28 ± 3.79 ab	1.12 ± 0.21 a	14.35 ± 1.06 ab	12.98 ± 1.32 a	11.29 ± 1.01 a	1.11 ± 0.11 c	1.28 ± 0.36 a
	Ani	42.80 ± 3.40 bcd	1.57 ± 0.20 c	15.53 ± 1.28 e	15.55 ± 1.18 d	13.06 ± 0.95 cde	1.00 ± 0.10 ab	1.74 ± 0.54 bc
	SA	41.26 ± 2.37 abc	1.38 ± 0.23 b	14.54 ± 1.24 abc	13.74 ± 1.04 b	12.12 ± 1.10 b	1.06 ± 0.12 bc	1.64 ± 0.34 b
	SAi	44.42 ± 3.16 d	1.63 ± 0.20 c	15.15 ± 1.17 cde	15.47 ± 0.96 d	13.67 ± 0.87 e	0.98 ± 0.08 a	1.86 ± 0.42 b
	P	***	***	***	***	***	***	***
2017	C	41.50 ± 5.21 ab	1.08 ± 0.17 a	13.57 ± 0.81	13.24 ± 1.40 a	11.39 ± 1.15 a	1.04 ± 0.14 a	1.16 ± 0.37 a
	I	42.57 ± 2.57 ab	1.40 ± 0.14 bcd	14.32 ± 0.75	14.29 ± 0.89 b	12.93 ± 1.08 b	1.01 ± 0.08 a	1.84 ± 0.37 c
	K	43.16 ± 4.26 b	1.37 ± 0.20 bc	14.57 ± 0.90	14.20 ± 1.14 b	12.60 ± 0.84 b	1.03 ± 0.11 a	1.60 ± 0.50 b
	Ki	43.35 ± 3.72 ab	1.44 ± 0.19 bcd	15.25 ± 0.76	14.64 ± 1.08 b	13.03 ± 1.43 b	1.01 ± 0.22 a	1.95 ± 0.15 c
	An	39.69 ± 3.90 a	1.14 ± 0.22 a	14.26 ± 0.94	12.78 ± 1.47 a	11.19 ± 1.35 a	1.13 ± 0.13 b	1.18 ± 0.39 a
	Ani	44.07 ± 2.36 b	1.51 ± 0.21 d	15.28 ± 1.06	14.52 ± 1.00 b	13.02 ± 0.82 b	1.06 ± 0.07 ab	1.76 ± 0.44 bc
	SA	42.00 ± 2.94 ab	1.47 ± 0.14 cd	15.13 ±0.84	14.36 ± 1.12 b	12.64 ± 1.22 b	1.06 ± 0.12 ab	1.96 ± 0.20 c
	SAi	44.71 ± 4.36 b	1.35 ± 0.22 b	14.50 ± 0.85	14.18 ± 1.20 b	12.43 ± 1.12 b	1.03 ± 0.09 a	1.88 ± 0.34 c
	P	***	***	n.s	***	***	*	***
	Year (Y)	n.s.	**	n.s.	**	n.s.	n.s.	n.s.
	Treatment (T)	***	***	n.s.	***	***	***	***
	Y x T	n.s.	**	n.s.	**	**	n.s.	n.s.

[1]Values expressed as mean ± standard deviation (SD). Abbreviation means: (C)-Control; (I)-Irrigation; (K)-Kaolin; (Ki)-Kaolin with irrigation; (An)-*Ascophyllum nodosum*; (Ani)-*Ascophyllum nodosum* with irrigation; (SA)-Salicylic Acid and (SAi)-Salicylic Acid with irrigation.
[2]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at P<0.05 by Tukey test.
[3]The symbols means: n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001.

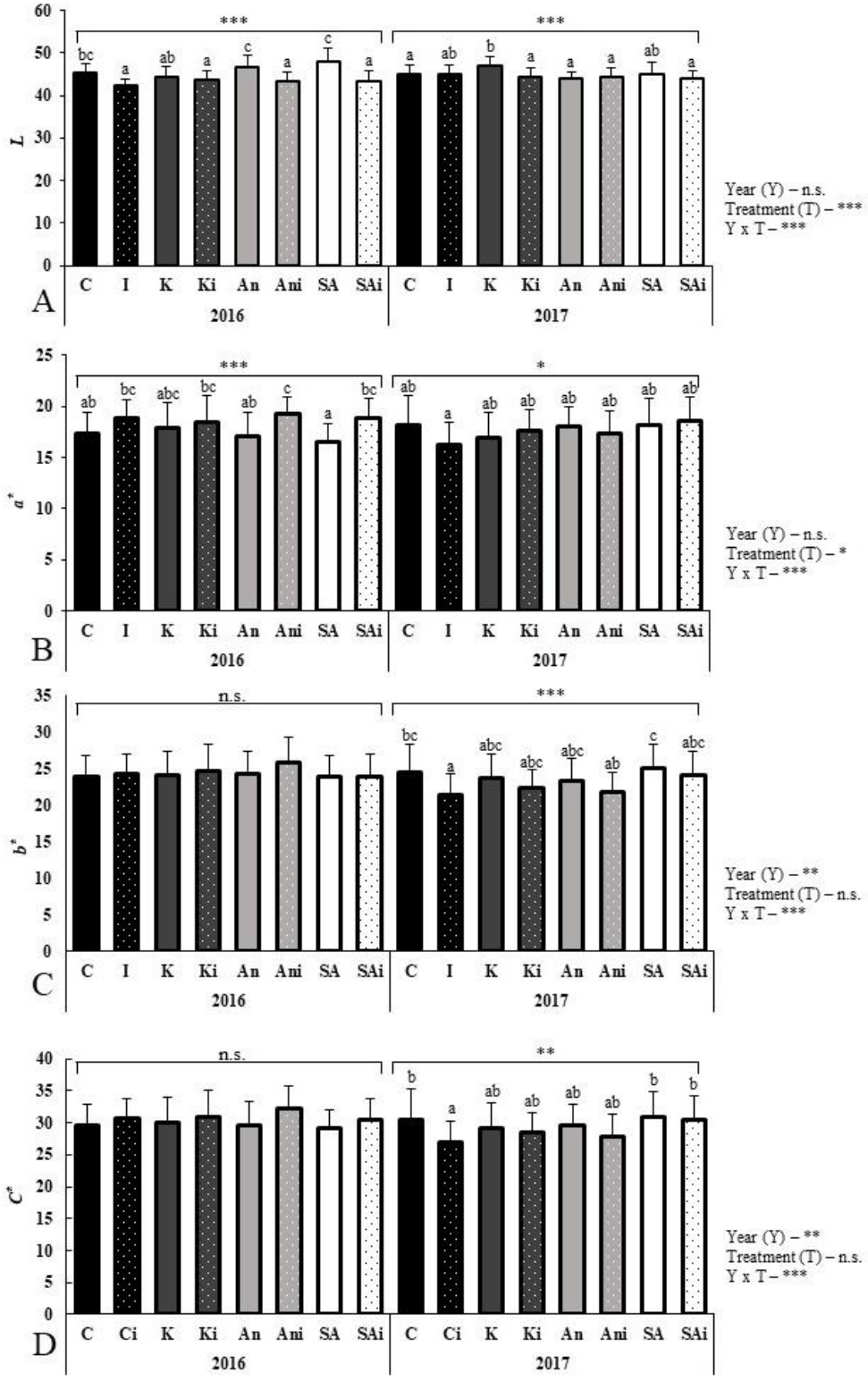

Figure 5.1. Colorimetric parameters of nuts with different treatments (C-Control; I-Irrigation; K-Kaolin; Ki-Kaolin with irrigation; An-*Ascophyllum nodosum*; Ani-*Ascophyllum nodosum* with irrigation; SA-Salicylic Acid and SAi-Salicylic Acid with irrigation) in two consecutive years (2016 and 2017). Values are expressed as mean ± SE. Results from ANOVA analysis are expressed by the symbols n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001. Different letters above each bar of the graph represents significant differences (P<0.05) by Tukey test.

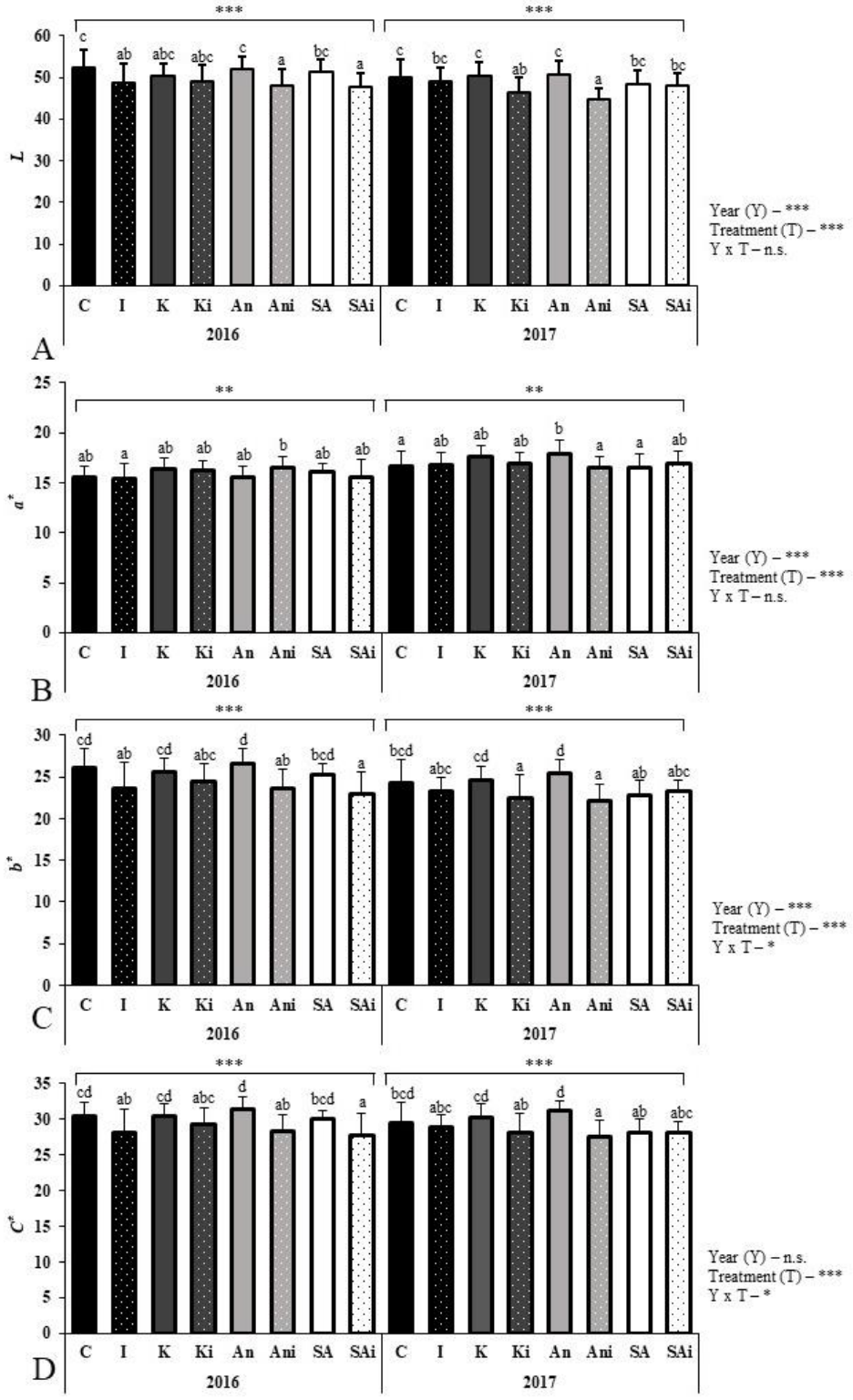

Figure 5.2. Colorimetric parameters of kernels with different treatments (C-Control; I-Irrigation; K-Kaolin; Ki-Kaolin with irrigation; An-*Ascophyllum nodosum*; Ani-*Ascophyllum nodosum* with irrigation; SA-Salicylic Acid and SAi-Salicylic Acid with irrigation) in two consecutive years (2016 and 2017). Values are expressed as mean ± SE. Results from ANOVA analysis are expressed by the symbols n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001. Different letters above each bar of the graph represents significant differences (P<0.05) by Tukey test.

5.4.2. Kernel chemical and phytochemical composition

Significant differences were found for amino acids ($P<0.05$) (Table 5.4.), for TPC, TFC ($0.05<P<0.001$) (Figure 5.3.) and for Vit. E, TS and St ($P<0.001$) (Table 5.5.), between treatments and year of experiment. Nine different free amino acids were identified in, being glutamic acid (Glu), arginine (Arg), alanine (Ala) and Aspartic acid (Asp) the four main amino acids found. The amino acid levels were lower in all treatments compared to control. Regarding the content of different classes of polyphenols there was not any consistent trend between years, but in general, kernels from trees treated with K in the second year of experiment presented the highest significant content of TPC and TFC (Figure 5.3.). while the treatment of SAi recorded the highest significant content of o-DP. This tendency was corroborated partially with individual phenolic composition (Table 5.6.). Eleven compounds were found grouped into four main classes: hydroxybenzoic acids (gallic and protocatechuic acids), hydroxycinnamic acids (neochlorogenic acid), flavan-3-ols (gallocatechin, epigallocatechin, catechin, epicatechin, epigallocatechin gallate and gallocatechin gallate) and flavonols (myric-3-*O*-ramnoside and its respective isomer). In general, year and treatments significantly affected ($P<0.001$) each phenolic level. In general, lower concentration of the most phenolic compounds was observed than those of control in the second year, except of procatechuic acid in K treatment and (-)-epigallocatechin in An treatment (Table 5.6.).

Table 5.4. Content of amino acids in kernels with different treatments, collected in two consecutive years (2016 and 2017) from an orchard in Moimenta da Beira Region, north of Portugal.[1-3]

Year	Treatment	Aspartic acid (Asp)	Glutamic acid (Glu)	Asparagine (Asn)	Glutamine (Gln)	Glycine (Gly)	Threonine (Thr)	Arginine (Arg)	Alanine (Ala)	Tyrosine (Tyr)
2016	C	1.44 ± 0.12 f	4.70 ± 0.30 d	0.69 ± 0.04 d	0.89 ± 0.16 d	0.52 ± 0.03 d	0.79 ± 0.10 c	3.65 ± 0.11 d	0.39 ± 0.07 a	0.76 ± 0.04 c
	I	0.53 ± 0.01 a	2.71 ± 0.03 b	0.16 ± 0.05 a	0.30 ± 0.05 abc	0.11 ± 0.01 a	0.45 ± 0.01 b	1.30 ± 0.99 bc	1.12 ± 0.93 e	0.50 ± 0.02 b
	K	1.03 ± 0.02 d	3.29 ± 0.03 c	0.14 ± 0.02 a	0.47 ± 0.00 c	0.12 ± 0.01 a	0.20 ± 0.04 a	0.34 ± 0.01 ab	0.82 ± 0.04 c	0.49 ± 0.02 b
	Ki	0.73 ± 0.02 b	2.35 ± 0.10 ab	0.18 ± 0.01 ab	0.24 ± 0.00 ab	0.10 ± 0.01 a	0.23 ± 0.00 a	0.24 ± 0.07 a	0.64 ± 0.07 b	0.24 ± 0.04 a
	An	1.21 ± 0.02 e	3.13 ± 0.11 c	0.27 ± 0.01 b	0.35 ± 0.03 abc	0.20 ± 0.02 b	0.28 ± 0.06 a	0.67 ± 0.02 abc	0.95 ± 0.03 d	0.84 ± 0.03 c
	Ani	0.89 ± 0.03 c	2.45 ± 0.01 b	0.49 ± 0.04 c	0.42 ± 0.12 bc	0.61 ± 0.02 e	0.25 ± 0.04 a	1.45 ± 0.22 c	0.88 ± 0.02 cd	0.77 ± 0.06 c
	SA	0.92 ± 0.02 cd	2.46 ± 0.08 b	0.17 ± 0.01 a	0.28 ± 0.01 abc	0.36 ± 0.06 c	0.75 ± 0.07 c	0.26 ± 0.04 a	0.60 ± 0.00 b	0.51 ± 0.04 b
	SAi	0.72 ± 0.02 b	2.08 ± 0.08 a	0.10 ± 0.00 a	0.17 ± 0.02 a	0.09 ± 0.03 a	0.20 ± 0.00 a	0.25 ± 0.05 a	0.60 ± 0.02 b	0.48 ± 0.02 b
	P	***	***	***	***	***	***	***	***	***
2017	C	1.06 ± 0.03 e	3.18 ± 0.06 e	0.37 ± 0.04 e	0.69 ± 0.01 b	0.77 ± 0.11 de	0.47 ± 0.02 ab	0.86 ± 0.09 abc	1.15 ± 0.01 bc	0.74 ± 0.08 a
	I	0.61 ± 0.03 a	2.84 ± 0.03 d	0.33 ± 0.01 de	0.66 ± 0.11 b	0.84 ± 0.01 e	0.65 ± 0.08 bc	0.77 ± 0.21 ab	1.65 ± 0.11 e	1.28 ± 0.01 bc
	K	1.03 ± 0.04 e	2.72 ± 0.03 cd	0.49 ± 0.00 f	0.60 ± 0.08 b	0.67 ± 0.04 cde	0.65 ± 0.05 bc	0.75 ± 0.13 ab	1.29 ± 0.11 cd	1.31 ± 0.28 c
	Ki	0.59 ± 0.03 a	2.61 ± 0.10 bc	0.11 ± 0.02 a	0.33 ± 0.08 a	0.10 ± 0.00 a	0.22 ± 0.01 a	0.68 ± 0.03 a	0.61 ± 0.03 a	0.72 ± 0.08 a
	An	0.94 ± 0.00 d	3.22 ± 0.04 e	0.28 ± 0.00 c	0.51 ± 0.05 ab	0.66 ± 0.02 cd	0.71 ± 0.03 bc	1.32 ± 0.07 c	1.05 ± 0.03 b	0.90 ± 0.11 ab
	Ani	0.53 ± 0.02 a	1.53 ± 0.06 a	0.15 ± 0.13 a	0.32 ± 0.05 a	0.20 ± 0.04 a	0.37 ± 0.02 a	1.02 ± 0.03 abc	1.00 ± 0.04 b	0.73 ± 0.08 a
	SA	0.83 ± 0.03 c	2.88 ± 0.04 d	0.31 ± 0.01 cd	0.50 ± 0.09 ab	0.55 ± 0.11 bc	0.83 ± 0.01 c	1.18 ± 0.37 bc	1.38 ± 0.03 d	0.95 ± 0.24 abc
	SAi	0.71 ± 0.04 b	2.47 ± 0.13 b	0.19 ± 0.01 b	0.45 ± 0.15 ab	0.48 ± 0.04 b	0.70 ± 0.22 bc	0.79 ± 0.01 ab	1.11 ± 0.04 b	0.85 ± 0.01 a
	P	***	***	***	***	***	***	**	***	***
	Year (Y)	***	***	n.s.	***	***	*	n.s.	***	***
	Treatment (T)	***	***	***	***	***	***	***	***	***
	Y x T	***	***	***	***	***	***	***	***	***

[1]Values expressed as mean ± standard deviation (SD) of three replicates. Abbreviation means: (C)-Control; (I)-Irrigation; (K)-Kaolin; (Ki)-Kaolin with irrigation; (An)-*Ascophyllum nodosum*; (Ani)-*Ascophyllum nodosum* with irrigation; (SA)-Salicylic Acid and (SAi)-Salicylic Acid with irrigation.
[2]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at P<0.05 by Tukey test.
[3]The symbols means: n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001.

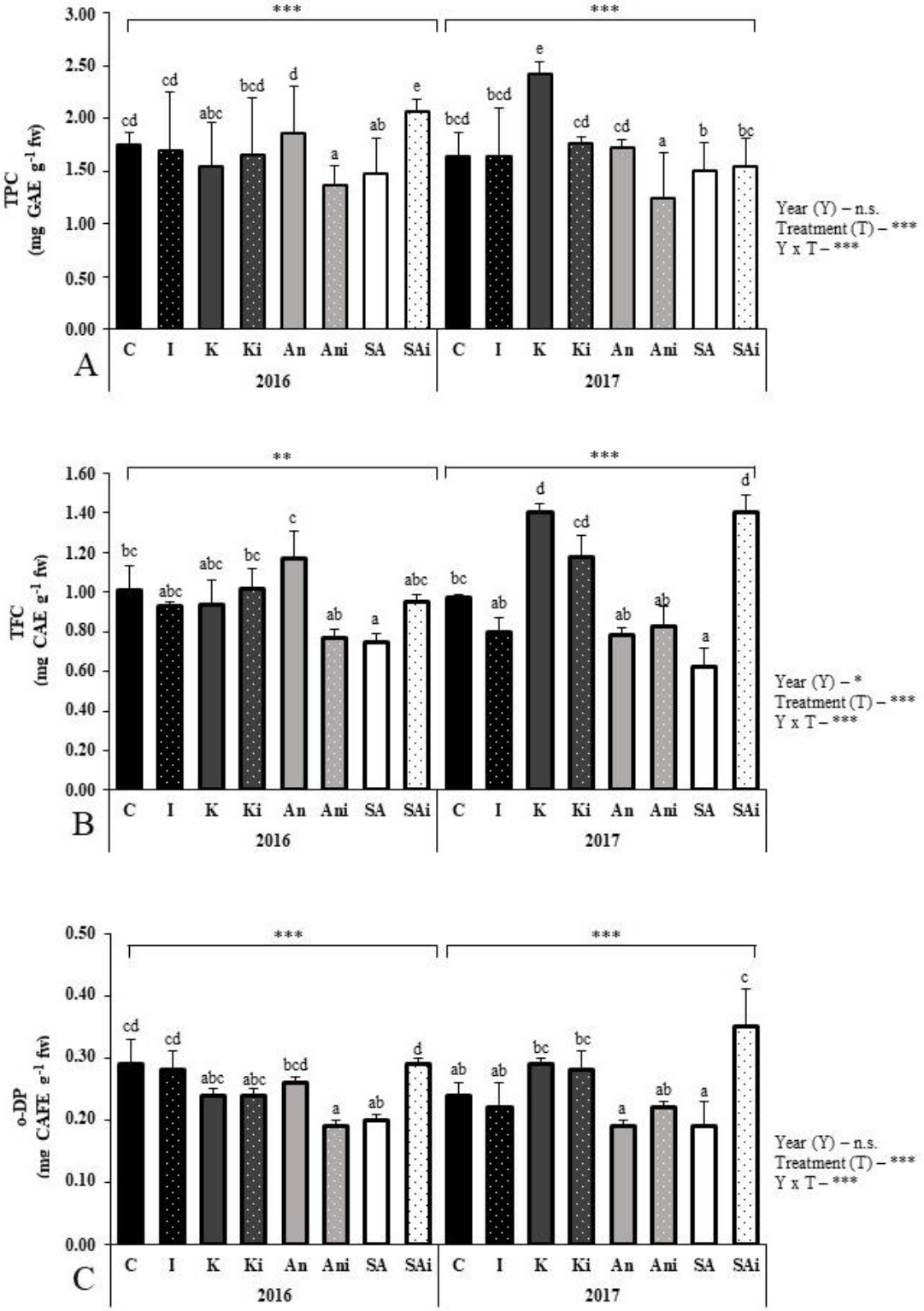

Figure 5.3. Variation on chemical parameters (A) TPC-Total Phenolic Content, (B) TFC-Total Flavanoid and (C) *o*-DP-*ortho*-Diphenols in kernels with different treatments (C-Control; I-Irrigation; K-Kaolin; Ki-Kaolin with irrigation; An-*Ascophyllum nodosum*; Ani-*Ascophyllum nodosum* with irrigation; SA-Salicylic Acid and SAi-Salicylic Acid with irrigation) in two consecutive years (2016 and 2017). Values are expressed as mean ± SE. Results from ANOVA analysis are expressed by the symbols n.s. $P>0.05$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Different letters above each bar of the graph represents significant differences ($P<0.05$) by Tukey test.

Table 5.5. Variation on Vit. E, TS and St in kernels with different treatments, collected in two consecutive years (2016 and 2017) from and orchard in Moimenta da Beira Region, north of Portugal.[1-3]

Year	Treatment	Vit. E ($\mu g\ g^{-1}$ fw)	TS (mg glucose g^{-1} fw)	St (mg g^{-1} fw)
2016	C	93.78 ± 3.12 a	32.91 ± 3.58 b	57.13 ± 3.35 de
	I	147.67 ± 16.59 b	33.68 ± 0.43 b	58.60 ± 3.63 e
	K	148.13 ± 4.67 b	55.29 ± 4.58 c	49.13 ± 2.40 cd
	Ki	191.71 ± 4.24 c	35.96 ± 1.03 b	52.60 ± 3.25 de
	An	218.42 ± 23.54 c	34.73 ± 0.97 b	57.41 ± 3.94 de
	Ani	149.93 ± 3.39 b	30.55 ± 0.53 b	43.47 ± 3.24 bc
	SA	150.86 ± 1.07 b	32.58 ± 1.59 b	33.24 ± 1.57 a
	SAi	206.39 ± 3.94 c	23.26 ± 0.70 a	38.19 ± 2.76 ab
	P	***	***	***
2017	C	171.78 ± 15.95 abc	47.26 ± 2.40 d	38.07 ± 1.86 bc
	I	144.86 ± 3.57 ab	39.64 ± 1.57 c	38.23 ± 2.17 bc
	K	185.64 ± 15.55 c	27.49 ± 0.80 ab	44.69 ± 3.09 c
	Ki	156.51 ± 8.01 abc	29.67 ± 1.38 ab	37.37 ± 2.04 b
	An	179.58 ± 8.95 bc	45.68 ± 1.36 d	33.62 ± 0.59 ab
	Ani	173.37 ± 15.89 abc	35.81 ± 1.71 c	29.51 ± 0.75 a
	SA	139.90 ± 1.99 a	27.04 ± 0.83 a	35.01 ± 2.81 ab
	SAi	222.78 ± 17.94 d	31.50 ± 1.05 b	33.28 ± 3.83 ab
	P	***	***	***
Year (Y)		*	n.s.	***
Treatment (T)		***	***	***
Y x T		***	***	***

[1]Values expressed as mean ± standard deviation (SD). Abbreviations and numbers: (Vit. E)-Vitamin E; (TS)-Total Sugars; (St)-Starch; (C)-Control; (I)-Irrigation; (K)-Kaolin; (Ki)-Kaolin with irrigation; (An)-*Ascophyllum nodosum*; (Ani)-*Ascophyllum nodosum* with irrigation; (SA)-Salicylic Acid and (SAi)-Salicylic Acid with irrigation.
[2]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at P<0.05 by Tukey test.
[3]The symbols means: n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001.

Table 5.6. Content of phenolic compounds quantified in kernels with different treatments, collected in two consecutive years (2016 and 2017) from an orchard in Moimenta da Beira Region, north of Portugal (values expressed as mg g^{-1} fw).[1-3]

Year	Treatment	Gallic acid	Protocatechuic acid	Neochlorogenic acid	(-)-Gallocatechin	(-)-Epigallocatechin	(+)-Catechin	(-)-Epicatechin	(-)-Epigallocatechin gallate	(-)-Gallocatechin gallate	Myric-3-*O*-ramnoside	Myrecetin isomer
2016	C	15.35 ± 0.46	33.54 ± 0.73 ab	1.50 ± 0.23 ab	44.54 ± 3.97 ab	11.16 ± 2.89 ab	37.21 ± 6.80 ab	47.46 ± 5.52 bcd	18.87 ± 5.29	13.40 ± 0.11 a	3.31 ± 1.39	0.93 ± 0.32
	Ci	16.00 ± 1.03	43.04 ± 4.79 bc	1.78 ± 0.22 bc	50.91 ± 0.95 ab	17.36 ± 3.04 b	40.68 ± 3.36 ab	59.97 ± 5.29 d	19.57 ± 2.71	19.55 ± 1.48 ab	2.83 ± 0.17	0.88 ± 0.10
	K	16.82 ± 1.11	49.70 ± 4.04 cd	2.05 ± 0.22 c	70.59 ± 4.55 c	15.83 ± 0.88 b	48.34 ± 4.67 bc	51.10 ± 3.08 cd	22.22 ± 1.35	22.65 ± 2.82 b	2.39 ± 0.28	0.60 ± 0.07
	Ki	13.46 ± 1.71	39.27 ± 4.35 ab	1.53 ± 0.12 ab	47.27 ± 6.28 ab	15.91 ± 3.83 b	37.99 ± 5.38 ab	37.36 ± 5.59 ab	18.22 ± 3.11	17.33 ± 2.75 ab	2.68 ± 0.66	0.84 ± 0.18
	An	16.56 ± 1.82	57.67 ± 3.26 d	1.65 ± 0.11 abc	66.60 ± 3.33 c	7.05 ± 1.70 a	63.21 ± 8.05 c	45.01 ± 4.64 abcd	23.37 ± 3.26	18.48 ± 5.58 ab	3.38 ± 0.25	0.75 ± 0.07
	Ani	13.96 ± 2.74	29.44 ± 3.93 a	1.22 ± 0.06 a	39.48 ± 7.45 a	9.05 ± 1.34 ab	31.23 ± 5.01 a	33.07 ± 5.78 a	16.30 ± 1.70	15.37 ± 1.64 ab	2.37 ± 0.63	0.70 ± 0.17
	SA	12.53 ± 0.79	41.43 ± 3.76 bc	2.03 ± 0.13 c	53.50 ± 2.75 b	14.32 ± 2.51 ab	35.51 ± 3.94 ab	41.37 ± 5.02 abcd	18.52 ± 2.65	17.72 ± 1.23 ab	2.36 ± 0.49	0.57 ± 0.12
	SAi	14.04 ± 1.40	39.38 ± 1.69 ab	1.22 ± 0.18 a	44.89 ± 2.77 ab	12.24 ± 5.27 ab	36.99 ± 3.51 ab	38.50 ± 0.43 abc	18.85 ± 3.61	15.77 ± 0.36 ab	3.01 ± 0.37	0.94 ± 0.18
	P	n.s.	***	***	***	**	***	**	n.s.	*	n.s.	n.s.
2017	C	32.29 ± 2.11 bc	75.89 ± 4.04 bc	3.74 ± 0.40 bc	112.77 ± 9.77 b	11.28 ± 1.22 a	69.13 ± 15.28 cd	71.17 ± 12.33 b	69.13 ± 15.28	25.21 ± 3.79 ab	5.64 ± 1.54	1.66 ± 0.48 b
	Ci	37.10 ± 3.99 c	69.88 ± 5.77 b	3.81 ± 0.43 c	95.46 ± 11.08 b	8.40 ± 3.30 a	47.13 ± 11.24 abc	63.22 ± 8.00 ab	47.13 ± 11.24	25.94 ± 3.76 ab	5.43 ± 1.66	1.68 ± 0.49 b
	K	30.84 ± 2.64 bc	82.65 ± 8.01 c	2.96 ± 0.41 abc	108.85 ± 11.16 b	9.48 ± 0.71 a	66.81 ± 5.30 bcd	53.39 ± 14.36 ab	66.81 ± 5.30	20.91 ± 1.86 a	4.51 ± 0.49	1.35 ± 0.15 ab
	Ki	27.97 ± 1.92 ab	67.25 ± 2.54 b	3.24 ± 0.22 bc	97.04 ± 2.11 b	10.34 ± 2.06 a	71.27 ± 2.90 d	46.16 ± 1.18 a	71.27 ± 2.90	28.82 ± 3.57 b	6.04 ± 0.42	1.91 ± 0.10 b
	An	32.10 ± 1.15 bc	72.16 ± 2.12 bc	3.16 ± 0.27 bc	101.52 ± 4.58 b	23.20 ± 3.38 b	52.12 ± 4.18abcd	70.31 ± 5.27 ab	52.12 ± 4.18	31.12 ± 2.21 b	4.74 ± 0.65	0.72 ± 0.10 a
	Ani	27.58 ± 3.07 ab	64.92 ± 4.00 b	2.88 ± 0.18 ab	93.29 ± 4.21 b	19.04 ± 2.32 b	37.00 ± 5.03 a	49.51 ± 4.35 ab	37.00 ± 5.03	30.02 ± 1.42 b	5.02 ± 0.50	1.26 ± 0.12 ab
	SA	33.20 ± 1.28 bc	49.51 ± 3.22 a	3.55 ± 0.27 bc	96.89 ± 8.03 b	7.38 ± 2.51 a	55.13 ± 11.41 abcd	62.69 ± 11.21 ab	55.13 ± 11.41	24.31 ± 3.07 ab	3.92 ± 0.64	1.26 ± 0.17 ab
	SAi	23.09 ± 0.93 a	46.57 ± 1.88 a	2.20 ± 0.24 a	68.22 ± 1.64 a	10.76 ± 1.57 a	44.29 ± 0.93 ab	47.40 ± 4.73 ab	44.29 ± 0.93	19.74 ± 1.11 a	4.52 ± 0.08	1.33 ± 0.10 ab
	P	***	***	***	***	***	**	*	n.s.	**	n.s.	**
	Year (Y)	***	***	***	***	n.s.	***	***	***	***	***	***
	Treatment (T)	***	***	***	***	n.s.	***	***	n.s.	**	n.s.	***
	Y x T	***	***	**	***	***	***	n.s.	n.s.	***	n.s.	*

[1]Values expressed as mean ± standard deviation (SD). Abbreviation means: (C)-Control; (I)-Irrigation; (K)-Kaolin; (Ki)-Kaolin with irrigation; (An)-*Ascophyllum nodosum*; (Ani)-*Ascophyllum nodosum* with irrigation; (SA)-Salicylic Acid and (SAi)-Salicylic Acid with irrigation.
[2]Probability test values obtained by ANOVA variance analysis; number with different letters in same column are significantly different from another at P<0.05 by Tukey test.
[3]The symbols means: n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001.

5.4.3. Antiradical and antioxidant activity

The bioactive properties of hazelnut kernels based in its antiradical and antioxidant activities, measured by three different methods, showed significant differences ($P<0.001$) between years and treatments (Figure 5.4.). In general, higher antiradical and antioxidant activities were observed in kernels from trees treated with K, Ki, An, SA (Figure 5.4.). The trend of antioxidant activities was very similar between the three methods assayed and averages values exhibited were higher in 2017, in all three methods. Similarly, the DPPH values for K, Ki and An were comparable to TPC and TFC, and FRAP values (Figure 5.3.). While the values of the Inhibition of Lipid Peroxidation (Figure 5.4.) seem to follow the variation of other compounds, including TPC and TFC, since the increment of antioxidant activities under An was higher compared with Ki and similar to K.

5.4.4. Sensorial analysis

Sensorial analyses of kernels showed that year and treatment have no significant effect ($P>0.05$) in the overall sensory attributes evaluated by panelists. Significant differences were only found in colour attribute for the fruits collected in 2017. Similar values in very positive attributes such as *flavour*, *integrity of tegument*, *hardness* and *sweetness* of kernels from different treatments were indicated by the panelists (Figure 5.5). Moreover, the intensity of the *rancid aroma* was very low, in both years, not exceeding 1.5 on the scale.

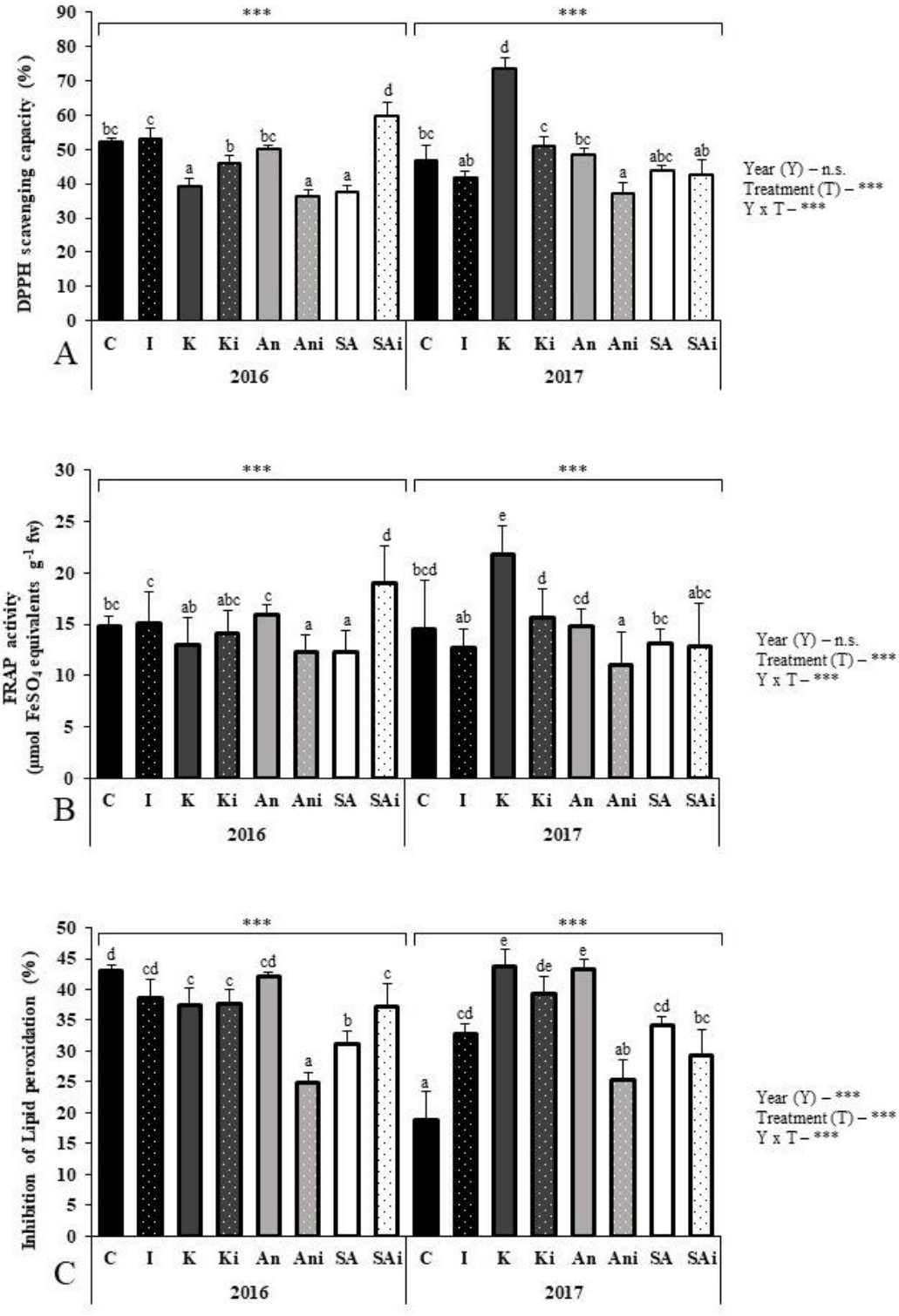

Figure 5.4. Antioxidant activity (A) DPPH scavenging capacity, (B) FRAP activity and (C) Inhibition of lipid peroxidation content in kernels with different treatments (C-Control; I-Irrigation; K-Kaolin; Ki-Kaolin with irrigation; An-*Ascophyllum nodosum*; Ani-*Ascophyllum nodosum* with irrigation; SA-Salicylic Acid and SAi-Salicylic Acid with irrigation) in two consecutive years (2016 and 2017). Values are expressed as mean ± SE. Results from ANOVA analysis are expressed by the symbols n.s. P>0.05, * P<0.05, ** P<0.01, *** P<0.001. Different letters above each bar of the graph represents significant differences (P<0.05) by Tukey test.

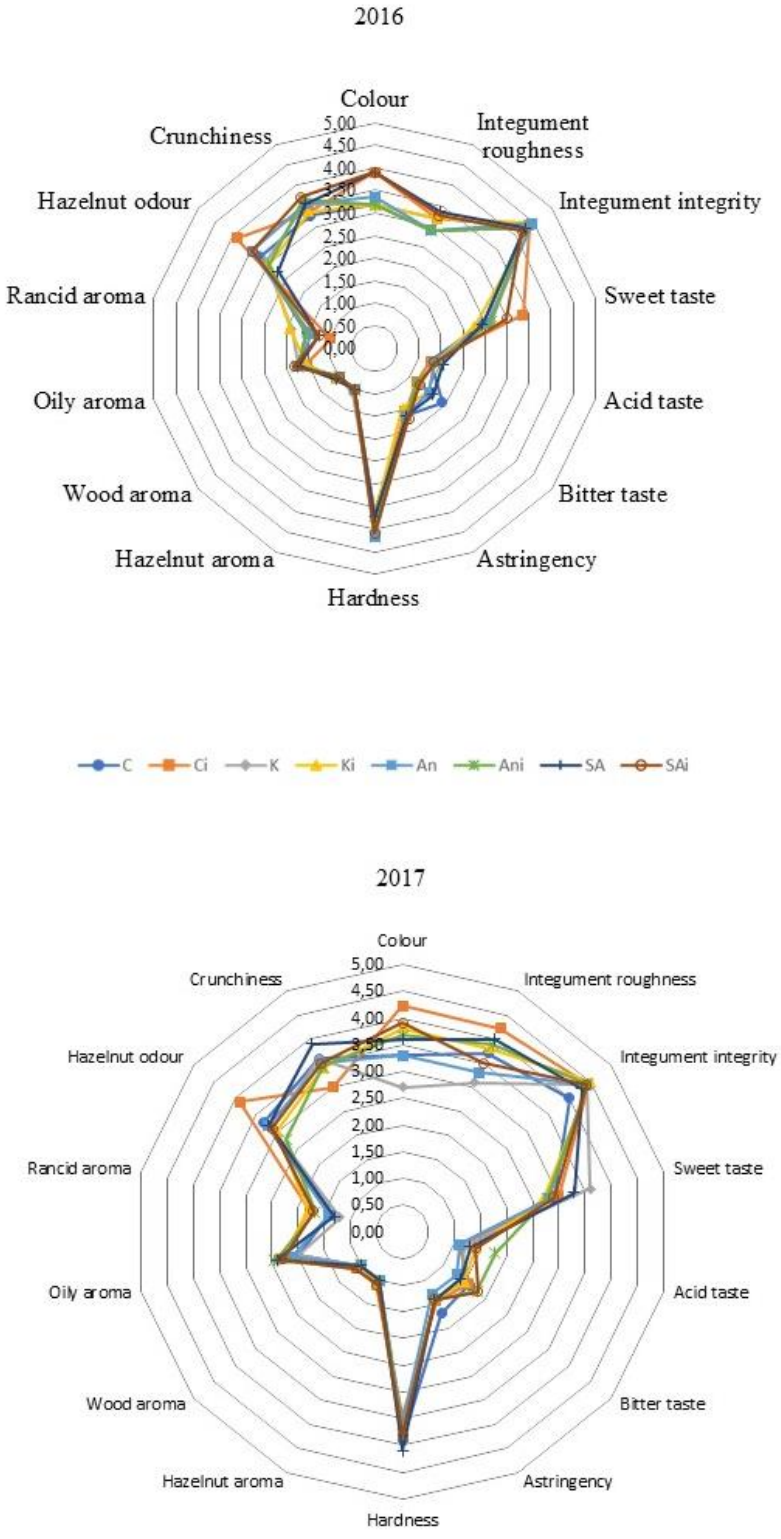

Figure 5.5. Spider plot of sensory profile of kernels with different treatments (C-Control; I-Irrigation; K-Kaolin; Ki-Kaolin with irrigation; An-*Ascophyllum nodosum*; Ani-*Ascophyllum nodosum* with irrigation; SA-Salicylic Acid and SAi-Salicylic Acid with irrigation) in two consecutive years (2016 and 2017). Values are expressed as mean ± SE. Results from ANOVA analysis (Duncan´s test) are expressed by the symbols * $P<0.05$. Absence of superscript indicates no significant differences.

5.5. Discussion

5.5.1. Physical attributes and harvest quality of hazelnuts (shells and kernels) were positively influenced by year and spray treatments

It is well known by literature that climate conditions and cultural practices influence strongly the crop productivity, as well as the fruit chemical and physical attributes. Patterns in climate can affect not only the physiological performance of trees but also its main phenological stages (Kappelle et al., 1999; Li et al., 2017; Tixier et al., 2020), and thus interfering directly in the production and productivity every year. Considering the monthly temperatures and precipitation throughout the two years of experiment (Table 5.1), it can be stated that 2017 was drier and hotter than 2016, particularly from April to June, when fruit set occurred in hazelnut trees, which may justify the lower yield per tree obtained in 2017. Less water and higher temperature, during this important phenological stage, induce abiotic stress in trees, reducing metabolic processes and thus lowering flower differentiation, and consequently reducing the fruit set (Waraich et al., 2012; Baldwin, 2015). As consequence, fruit physical and chemical attributes are affected. However, external application of natural or synthetic compounds could mitigate this impact. Cantore et al. (2009), Ali et al. (2016) and Mohamed et al. (2018) reported positive effects of K, An and SA on the physical characteristics of tomato and strawberry. According to these authors, these substances are capable to increase solar reflection, photosynthetic efficiency, and intrinsic water use and to decrease foliar respiration rate, resulting in a protective effect for plants from summer stress. Brito et al. (2018) for olives and Cabo et al. (2019) for hazelnut trees, obtained similar findings. In the current study, it can be observed that external application K, An and SA, (with and without irrigation) in both years of experiment led the trees to preserve the physical attributes of nuts and kernels (Tables 5.2. and 5.3.). In fact, in these two years, nuts and kernels from trees treated with different treatments showed higher weight, volume, length, width and thickness, when compared to control fruits. In addition, irrigation induced significant differences in the physical attributes according to the treatment, particularly on second year of experiment, which could mean that in a medium to long-term situation the foliar sprayers with K, An and Sa, might be used as an alternative tool to irrigation. In addition, the similarity of results for *L*, a*, b* and C* for nuts and kernels (Figures 5.1. and 5.2.), in all treatments and both years, signify that foliar sprayers did not affect negatively the typical hazelnut colour attributes, which are a quality index for consumers and

industry acceptance (Donno et al., 2013; Sensory, 2019). Therefore, leaf sprayings with with K, An and SA can be used safely, without compromising the nut and kernel size or colour.

Although size and colour attributes are important in consumer's acceptance, in the fruit compositional quality, there are other primary aspects regarding the effect of external substances. Batista-Silva et al. (2019), recently reported that when plants are exposed to abiotic stresses, usually accumulate proteins and protein-related compounds such as free amino acid. Under drought stress, protein synthesis and protein turnover are enhanced to handle with water limitation conditions (Mohamed et al., 2018). Extensive increments in arginine, alanine, aspartate, glutamate, glycine, tyrosine was also observed by Michalleti et al. (2018) in Bahar leaves, as response of plants to higher drought conditions. According to these authors, plants under drought and heat stress, an osmotic adjustment occurs, causing a protein breakdown which leads to an amino acid accumulation. This is in agreement with the herein presented findings, in which a lower amino acid content was observed in kernels from trees treated with K, An and SA (with and without I), compared to C (Table 5.4.), meaning that irrigation and foliar sprayers with K, An and SA, were capable to reduce the tree stress, despite the lower content of amino acids. Nonetheless, independently of treatments it was found that for all kernels, four main free amino acids: aspartic acid, glutamic acid, arginine and alanine were present. These four amino acids have been reported in the literature as the four main amino acids in hazelnuts (Alasalvar et al., 2003).

Regarding the compositional quality of hazelnuts, the Vit. E content, as well as the TS and St content (Table 5.5.) was measured in this study. By the results, only a consistent trend was found for kernels from trees treated with SAi, which exhibited the highest level of Vit. E in both years of experiment. This could be related with the grade of stress exhibited by the trees under this treatment. Under stress, plants accumulate reactive oxygen species (ROS) which leads to an oxidative and cell damage (Munné-Bosh and Alegre, 2002) and thus, interfering in Vit. E biosynthetic pathways[40] through the decreasing of *p*-hydroxyphenylpyruvate dioxygenase (HPPD) and homogentisate phytyltransferase (HPT1) (Michaletti et al., 2018) enzyme activities (Norris et al.,1998; Collakova and DellaPenna, 2001). In this situation, metabolic pathways are disturbed, and Vit. E content decrease, meaning that by opposition in all situations that are capable to reduce plant stress are capable to preserve Vit. E metabolic biosynthetic pathways, and thus increasing its content in cells. In a study with canola (Metwally et al, 2018) SA was capable overcome the effect of stress induced by toxicity of boron, increasing the levels of α-tocopherol. The same authors verified that SA significantly boosted

the α-tocopherol contents in canola shoots under both B toxicity and non-B toxicity conditions, which seems to corroborate our findings, even if the objectives of this study are different. Therefore, according to our results, it can be assumed that under SAi application, plants are less stressed, and their fruits presented higher size, higher mass and higher content of Vit. E, despite the lower content in amino acids.

5.5.2. Phytochemical composition and antioxidant activity of hazelnut kernels are positively influenced by applied treatments

Besides physical attributes and chemical attributes, climate conditions and pre-harvest factors can affect the polyphenols biosynthesis in fruits, including hazelnuts (Amaral et al, 2005). Among cultural practices, foliar sprayers with different natural or synthetic compounds, can be included, even if their results are often non-consistent, less clear and somehow contradictory. For example, Brito et al. (Brito et al.,2018) did not found a specific trend about the effect of K and SA on polyphenol content in olives; rather, they report that climatic conditions are more important in the variation of these compounds. However, Conde et al. (Conde et al., 2016) found that K was capable to increase the accumulation of TF and anthocyanins in the latter ripening stage of grape berries, due to the stimulation of phenylpropanoid and flavonoid synthesis. Recently, Preciado-Rangel et al. (Preciado-Rangel et al.,2019) reported an increment of polyphenols (TPC and TFC) in cucumber, when SA was applied. They reported that SA induced the formation of hydrogen peroxide, and consequently stimulated the synthetize of polyphenols *via* increments in the activity of phenylalanine ammonium lyase. A similar effect of SA was reported in chili fruits by Sánchez-Chaves (Sánchez-Chávez et al.,2011). Also, Frioni et al. (Frioni et al., 2018) found that a natural bioestimulant containing *Ascophyllum nodosum* seaweed-extract was capable to increase the content of polyphenols in 'Sangiovese' grapes. In the present study, a non-consistent trend was observed in total (TPC, TFC and *o*-DP) (Figure 5.3.) and individual polyphenol (Table 6) between years and treatments. In the warmest year (2017), it was noticed that K and Ki treatments were able to accumulate highest concentrations of TPC, TF and o-DP. Similar findings were reported by Dinis et al. (2016) who observed a boost in antioxidant activity and phenolic content in berries and grapevine leaves when kaolin was applied to alleviate summer stress. According to these authors, kaolin not only promotes canopy photosynthesis but also, is

able to protect the photosystem II structure and its function and by consequence increase the partitioning of carbohydrates to the synthesis of antioxidant compounds, such as phenolics. Nonetheless, an irregular trend was observed in our study. This irregular trend in the variation of these three classes of polyphenols may be correlated not only with the foliar sprays applied and irrigation treatments adopted, but also with the role of each one in their respective biosynthesis, as reported by Ripoll et al. (2014). These authors, stated that in stressful conditions polyphenol biosynthesis can be affected in two different but interconnected ways: 1) the reduction of net photosynthesis that occurs in climate stressful conditions, resulting in a decrease of carbohydrate supply to the fruits, which is the major precursor of polyphenol biosynthesis, and 2) the stress conditions may exacerbate the oxidative stress, promoting the biosynthesis of this group of antioxidant compounds (Brito et al., 2018). However, it is still not clear what is the role of exogenous substances such as K, An and SA in the biosynthesis of polyphenols and how they can interact with climate conditions. Despite this unclear pattern, the main individual polyphenols found in hazelnut kernels were protocatechuic acid, gallocatechin, catechin and epicatechin (Table 5.6.), all often reported as having important bioactivities, particularly antioxidant, anti-obesity and anti-tumoral (Gupta et al., 2008). In this study, high values of antioxidant activity were found in samples from trees treated with K, An and SA, particularly in the second year as shown in Figure 5.4. The analysis of Pearson correlation (Table 5.7.), shows that antioxidant activities of hazelnut kernels, where highly correlated with variation in the content of polyphenols and less influence of Vit. E. Protocatechuic acid, catechin and epigallocatechin gallate were those with strong influence in antioxidant activity variation. Kernels from K, An and SA treatments, exhibited high content of these three main polyphenols as well as TPC, TFC and *o*-DP and high antioxidant activities. Therefore, based in our results, we can state that foliar sprays with K, An and SA, are capable to increase the content of some individual polyphenols, which increase the content of TPC, TFC and *o*-DP and by consequence enhance hazelnut kernels antioxidant activities.

Table 5.7. Pearson correlation coefficients of all phytochemical parameters with antioxidant capacity in kernels.[1-2]

Antioxidant activity	TPC	TFC	o-DP	Vit. E	1	2	3	4	5	6	7	8	9	10	11
DPPH	**0.943**	**0.516**	**0.554**	0.188	0.106	**0.330**	-0.009	0.165	-0.142	**0.390**	0.085	**0.350**	-0.139	0.121	0.161
	(0.000)	***(0.000)***	***(0.000)***	*(0.201)*	*(0.474)*	***(0.022)***	*(0.952)*	*(0.262)*	*(0.335)*	***(0.006)***	*(0.565)*	***(0.015)***	*(0.347)*	*(0.414)*	*(0.274)*
FRAP	**0.966**	**0.474**	**0.555**	0.270	0.049	**0.323**	-0.062	0.143	-0.176	**0.415**	0.041	**0.353**	-0.162	0.068	0.101
	(0.000)	***(0.001)***	***(0.000)***	*(0.064)*	*(0.742)*	***(0.025)***	*(0.674)*	*(0.332)*	*(0.230)*	***(0.003)***	*(0.784)*	***(0.014)***	*(0.272)*	*(0.648)*	*(0.497)*
ILp	**0.569**	0.134	0.220	-0.023	-0.150	-0.010	-0.218	-0.120	0.093	0.110	-0.115	0.046	-0.132	-0.164	-0.231
	(0.000)	*(0.365)*	*(0.132)*	*(0.875)*	*(0.309)*	*(0.947)*	*(0.137)*	*(0.417)*	*(0.531)*	*(0.455)*	*(0.434)*	*(0.759)*	*(0.370)*	*(0.266)*	*(0.115)*

[1]Values expressed as mean ± standard deviation (SD). Abbreviations means: (TPC)-Total Phenolic Contens; (TFC)-Total Flavanoids Contens; (o-DP)-Ortho-Diphenols; (Vit. E)-Vitamin E; (1)-Gallic acid; (2)- Protocatechuic acid; (3)- Neochlorogenic acid; (4)- (-)-Gallocatechin; (5)-(-)-Epigallocatechin; (6)- (+)-Catechin; (7)- (-)-Epicatechin (8)- (-)-Epigallocatechin gallate;; (9)- (-)-Gallocatechin gallate; (10)- Myric-3-*O*-ramnoside; (11)- Myrecetin isomer; (DPPH)-DPPH scavenging capacity; (FRAP)-FRAP activity and (ILp)-Inhibition of lipid peroxidation.
[2] In parenthesis is presented the value of P (P<0.05 *, P<0.01 ** and P<0.001 ***).

5.5.3. Spray treatments did not affect negatively the sensory attributes of kernels

In somehow, at the beginning of the work, it was expected an effect of K, An, and SA, with and without irrigation, in sensorial attributes of hazelnuts. Spray treatments and irrigation regime didn´t significantly affect ($P>0.05$) the sensorial attributes of kernels, as shown by the results expressed in the Figure 5.5. Only kernels from I showed a slightly brownish colour in 2017 compared to other treatments ($P<0.05$). All panellists reported similar scale attributes (Figure 5.5.) for all treatments, showing that treatments did not interfere with typical sensorial quality of hazelnut kernels. Therefore, K, An and SA, with and without irrigation, can be applied safely, without compromising the sensorial hazelnut kernel quality. Kernels for all treatments exhibited similar and typical characteristics of hazelnut aroma and odours, flavour and tastes, integument integrity, hardness, oily aroma and sweet taste.

5.6. Conclusion

A positive trend was observed between the variation of hazelnut physical properties (nut and kernels), chemical and phytochemical composition, antioxidant activities and sensorial properties (kernels) with foliar sprays of kaolin, *Ascophyllum nodosum* and salicylic acid, with and without irrigation treatments. In fact, the external application of kaolin, *Ascophyllum nodosum* and salicylic acid improved the hazelnut trees response to the climate variation between years without compromising the hazelnut quality. 'Grada de Viseu' nuts under these treatments presented heavier and bigger fruits, high vitamin E, sugars and polyphenols content, and antioxidant properties. On the other hand, compounds can be used as an alternative to irrigation in regions of water scarcity, to increase yield and nut quality. In the long-term climate change projections, kaolin, *Ascophyllum nodosum* and salicylic acid can be effective and cost-effective solutions to mitigate summer stress and contribute to a sustainable hazelnut production.

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Conflict of interest

The authors declare no conflict of interest.

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