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Original article

Hazelnut fruit and kernel traits: influence of training systems and harvest year

I. Oliveira[1], A.P. Silva[1], T. Santos[2], G. Igrejas[2,3] and B. Gonçalves[1]

[1] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[2] Functional Genomics and Proteomics Unit, Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[3] UCIBIO-REQUIMTE, Faculty of Science and Technology, New University of Lisbon, Monte da Caparica, Portugal

Summary

Hazelnut can be grown using different training systems, which can, ultimately, affect fruit parameters. This work intended to study the effect of two training systems – multi-stemmed shrub versus single trunk training – and harvest year, on fruit and kernel characteristics of two cultivars, 'Butler' and 'Segorbe'. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns, kernel phenolic content and antioxidant activity of those cultivars were determined as well. Although the training system was of significant importance for a considerable number of traits, the harvest year was the factor that influenced a higher number of the studied parameters. Additionally, the effect of each factor and their combination varied depending on the studied cultivar. Overall results indicated that, for 'Butler', multi-stemmed training appears to be more appropriate, while, for 'Segorbe', the single trunk training seems to be more suitable. These results clearly illustrate the direct influence of the training system, but also from the harvest year, on fruit characteristics of hazelnut, which should be considered when planting new orchards.

Keywords

Corylus avellana L., 'Butler' and 'Segorbe', multi-stem and single trunk training, fruit biometric characteristics, antioxidant activity, protein profile

Significance of this study

What is already known on this subject?

- The influence of canopy architecture and year in leaf characteristics and fruit morphological traits.

What are the new findings?

- Variation of fruit antioxidant capacity depending on the training system, but no influence on SDS-PAGE patterns.

What is the expected impact on horticulture?

- These results are a helpful tool when considering the planting of hazelnut orchards, to maximize production, but also to optimize fruit traits.

Introduction

European hazelnut, *Corylus avellana* L. is one of the most important nut crops, regarding worldwide production, with the countries of the Black Sea accounting for the majority of the production (Boccacci et al., 2013). From 2008 to 2012, and although the hazelnut orchards area has increased, both the production and the yield (1806.09 kg ha^{-1} to 1524.08 kg ha^{-1}, in 2008 and 2012, respectively) have presented a reduction (FAO, 2015). Like in many other plants, light intensity reaching the leaves of hazelnut can be improved by selecting the most suitable training system, influencing the physiological behaviour of the tree (Hampson et al., 2014), and, ultimately, become an effective way to optimize the production and quality of fruits (Gonçalves et al., 2009). Therefore, the canopy pruning has become a routine practice for fruit crops, leading to higher productivity (Hampson et al., 1996). If by one hand, in highlighted leaves, the changes occurring are to insure high photosynthetic capacity per unit leaf area (Frak et al., 2002), the changes occurring in shadowed leaves are to improve light interception (Niinemets et al., 1998). In hazelnut, that presents a natural bushing growth tendency, the most usual training system is the multi-stemmed shrub (Islam et al., 2005; Beyhan, 2007). However, this training system is associated with several inconveniences, including difficulty to control pests and diseases, the higher number of suckers, the increased complexity of harvesting, having fruit fall inside the shrub, and the low planting density (Silva et al., 2005) that, together with a long juvenile phase (5–7 unproductive years, without irrigation (Me et al., 2001), all decreasing productivity. In order to increase yield and to allow more effective cultural and technical practices, different training systems were developed, such as free single trunk, V or Y hedge shape (Silva et al., 2005). One of the most used alternative training systems in Portugal is the single trunk, as it allows a better and easier control of suckers and implementation of cultural and technical practices, providing the possibility for an increase of planting density, while facilitating the harvesting of fruits (Me et al., 2001). Previous studies are available regarding comparison of training systems (Germain and Sarraquigne, 1997; Islam et al., 2005; Me et al., 2001; Valentini et al., 2009), only one report, by our group, is available, comparing these training systems in two cultivars, 'Butler' and 'Segorbe' (Gonçalves et al., 2009), and referring to physiological characteristics of leaves, rather than fruit traits. 'Butler' is classified as a table variety, while 'Segorbe' has characteristics that make it suitable for industrial purposes (Ramalhosa et al., 2011). In this work we hypothesise that the training system – multi-stemmed shrub versus

single trunk training – used in two different hazelnut cultivars – 'Butler' and 'Segorbe', but also the harvest year, may influence the yield and several fruit characteristics, including morphological traits and antioxidant activity.

Materials and methods

Experimental trial

The study was conducted in adult plants of the hazelnut cultivars 'Butler' and 'Segorbe', in 2007, 2008 and 2014. The trees were grown as multi-stemmed shrubs or as a free single trunk (free vase), with a tree spacing of 5 × 3 m, in an experimental plot near Vila Real, northeast Portugal (latitude 41°19'N; longitude 7°44'W; altitude 470 m a.s.l.), planted in February 1984, with both training systems conducted in the same year. The climatic conditions are characterized as a transition from Csb to Csa (mesothermic climate with a partially dry summer) of Köppen (Kottek et al., 2006), with mean higher temperature ranging from 28.3°C to 31.6°C, and lower mean temperature from 2.8°C to 3.6°C. Average precipitation in the years of study varied between 800 mm to 950 mm. The orchard is located on a typical dystrochrept silt loam, fertilized and periodically drip-irrigated, with all agricultural practices performed in the same manner to all the trees.

Fruit analysis

Three trees per cultivar and training systems were selected, and the total fruit production was estimated by collecting and weighing the fruits (in 2007, 2008 and 2014). Three trees of each training system and two cultivars were randomly selected, and, in those, thirty fruits were harvested. Fruits were hand-picked from the ground at the beginning of the harvest in September, weighed, and width, length and thickness were measured using a caliper, and after de-shelling, the process was repeated on the kernel. Length was measured as the distance from the basal to the apical scar, width as the distance across the nut at its widest point and thickness as the maximum distance from front to back in a direction perpendicular to that for width. Fruit and kernel roundness index (RI) was calculated as the ratio between length and width:

$$(RI = \frac{Length}{Width}).$$

Fruit antioxidant activity

Extraction was performed as previously described by Ghirardello et al. (2006) with minor modifications. Previous to extraction, fruits (harvest of 2014) were air-dried to reduce moisture to the minimum, and kernels were grounded finely, and 1 g was extracted with 10 mL of 50% ethanol (v/v) in ultrapure water acidified with formic acid (pH 4), with constant stirring, for 77 minutes, at room temperature. Extracts were filtered using a 0.45 µm syringe filter and used for antioxidant assays. All extractions were done in triplicate (three extractions per tree, three trees per cultivar and training system) and readings done in duplicates. The determination of total phenolics was performed using the methodology described by Singleton and Rossi (1965) and quantification was achieved by spectrophotometrical readings at 765 nm, using gallic acid as standard. To determine the DPPH (2.2-diphenyl-1-picrylhydrazyl) radical scavenging assay was performed by the method described by Von Gadow et al. (1997), slightly modified. 75 microliters of sample extract were added to 3 mL of DPPH methanol solution (6×10^{-5} mol L^{-1}) and incubated for 1 h at room temperature in the dark. The absorbance was measured at 515 nm against a blank. The $ABTS^{\bullet+}$ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay was performed to evaluate the radical scavenging ability of crude extracts by the method of Re et al. (1999) with minor changes. $ABTS^{\bullet+}$ was generated by adding 88 µL of $K_2S_2O_8$ (140 mmol L^{-1}) to 5 mL of ABTS 7 mmol L^{-1} and incubated in the dark at room temperature for 12–16 h. This stock solution of $ABTS^{\bullet+}$ was diluted with ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm. 20 µL of extract or standard was mixed with 2 mL diluted $ABTS^{\bullet+}$ solution and after 6 minutes of reaction, absorbance value was measured at 734 nm with UV-vis spectrophotometer. The antioxidant capacity of the hazelnut extracts was also estimated by the FRAP (ferric reducing/antioxidant power) assay, in which the antioxidants present in the sample reduce the Fe(III)/tripyridyltriazine (TPTZ) complex to the blue ferrous form with an increase in absorbance at 593 nm. The FRAP assay used was adapted from the method developed by Benzie and Strain (1996). FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ in 40 mM hydrochloric acid and with 1 volume of 20 mM ferric chloride. A reagent blank reading was taken at 593 nm ($A_{reagent\ blank}$). Extracts or standard (0.2 mL) was added to this FRAP reagent (2 mL), and were incubated for 50 min. The absorbance was read (A_{sample}). Sample blank reading, using extract or standard (50 µL) and acetate buffer (2,150 µL), was taken too ($A_{sample\ blank}$). The difference between A_{sample} and $A_{sample\ blank}$ was calculated as $A_{1sample}$. The FRAP value was calculated according the change in absorbance of $A_{1sample}$ against $A_{reagent\ blank}$. Results for DPPH, ABTS and FRAP assays were expressed as millimoles of Trolox equivalent (TE) per kilogram of sample, by means of a dose-response curve for Trolox (0–1,000 µM).

Protein extraction and 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis

Kernels were grounded and the grain flours of hazelnut fruits (harvest of 2014) were properly stored and identified in individual tubes. Two sets of tubes (set A and set B) were prepared with 0.05 g of flour each. A protocol for the extraction of high molecular weight (HMW) proteins was performed on the set A, and a protocol for the extraction of low molecular weight (LMW) proteins was conducted on the set B. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 15 µg of protein extract was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue. One-dimensional electrophoresis was conducted on vertical SDS-polyacrylamide gels (T=12.52%, C=0.97%) in a HoeferTMSE 600 Ruby® unit (Amersham Biosciences) according to Laemmli (1970). Proteins were separated with a constant current of 30 mA until the dye-front reached the bottom of the gel. Gels were then stained with Coomassie Brilliant Blue R250 for 24 h, then washed in water overnight. Gels were fixed in 6% TCA for 4 h then in 5% glycerol for 2 h (Gorg et al., 2000).

Data analysis

Data are presented as mean ± standard deviation, and differences among means were determined by analysis of variance (ANOVA), using SPSS (Statistical Package for Social Sciences) software, version 19.0 (IBM Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogor-

ov-Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro-Wilk's test (if $n < 50$), and the Levene's tests, respectively.

Results

Yield and fruit and kernel characteristics

In the 'Butler' cultivar, the harvested yield was considerably higher in multi-stemmed training (Table 1), while for 'Segorbe', higher yields were observed in the single trunk training (Table 2). For both cultivars, the harvest year also significantly influenced the yield, with higher values recorded in 2008. When analyzing results for fruit traits of 'Butler', no significant effect of the training systems was found for both weight and length (Table 1). However, when multi-stemmed training was used, the fruit presented higher width and thickness, with a lower roundness index (RI) (indicating a rounder fruit), than those recorded for the single trunk training. A significant effect of the harvest year was also found for these parameters. Interestingly, similar and higher values of weight, width and length were found in 2007 and 2008, while in 2014 values for these parameters were lower. On the other hand, fruit thickness and RI were not influenced by the harvest year. The interaction of the studied factors (training system and year) resulted in significant effects in almost all of the parameters concerning the fruit, with the exception of RI. The data from 'Segorbe' fruits indicate a dissimilar effect of the training system on fruit parameters (Table 2). Indeed, higher fruit weight was recorded for single trunk training, as well as fruit length, with higher values also recorded for RI. The year factor resulted in significant effects in all of the fruit parameters under analysis. For all parameters (weight, width, length, thickness and RI), values were higher in 2008, lower in 2014, with 2007 presenting in-between values. All fruit-related parameters were significantly affected by the interaction between all the studied factors. It must be pointed out that no significant effect of the interaction of factors was found for the harvest yield, although, in this specific parameter, data only refer to two years of study.

For kernel characteristics, and considering the data from 'Butler' (Table 3), it is found out that the training system significantly influenced the kernel/fruit weight ratio, with higher values recorded for multi-stemmed training. Contrarily, higher values of kernel length and RI were found in the single trunk training, while the other parameters were not influenced by this factor. The year factor also proved to exert significant influence on the dimensions (width, length and thickness) of 'Butler' kernels, as well as on RI. Among the fruits' dimensions, width and thickness were higher in 2014, but length reached the maximum value in 2007, being the lowest recorded in 2014. For RI, higher values were found in 2007 and 2008, with rounded kernels (lower RI) detected in 2014. Interestingly, the values for kernel weight were very similar and without significant differences between the studied years. The training system * year interaction resulted in significant variation of the kernel/fruit weight ratio, mainly due to the detected influence on the kernel weight. Statistically significant variations were also caused by the interaction of factors in the kernel width. For 'Segorbe' kernels, no significant effect caused by the training system was found, either in the kernel/fruit weight ratio, kernel weight or any of the dimensions, including RI. On the other hand, the year factor resulted in significant effects on almost all of those parameters, with the exception of the kernel length. A higher kernel/fruit weight ratio was found in 2014, although higher kernel weight was recorded in 2007. Kernel width and thickness present higher values also in 2007, although for the latter dimension, the recorded value was not statistically different from the value recorded in 2008. The kernel RI pre-

Table 1. Values (mean ± standard deviation) for yield and fruit characteristics of 'Butler' cultivar, and probability levels of the effects of Training System (multi-stemmed shrubs and free single trunk) and Year, as determined by two-way ANOVA. Probability levels: n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001. In bold, results that showed to be affected by the studied factors or their interaction.

		Yield (kg tree^{-1})	Fruit weight (g)	Fruit width (mm)	Fruit length (mm)	Fruit thickness (mm)	Fruit roundness index
Total		4880.7±3429.7	3.66±0.56	21.04±1.14	23.10±1.31	18.74±1.04	1.10±0.08
Training system (T)							
Multi-stemmed		**7435.7±2819.5**	3.66±0.55	**21.27±1.17 a**	22.93±1.25	**18.96±1.02 a**	**1.07±0.07 b**
Single trunk		**2325.7±1546.2**	3.66±0.56	**20.81±1.06 b**	23.27±1.35	**18.51±1.02 b**	**1.12±0.08 a**
Year (Y)							
2007		**3790.6±2664.9 b**	**3.83±0.58 a**	**21.44±1.27 a**	**23.79±1.08 a**	18.69±1.05	1.11±0.07
2008		**5789.1±3829.5 a**	**3.82±0.47 a**	**21.28±1.32 a**	**23.58±1.22 a**	18.84±1.19	1.10±0.08
2014		n.a.	**3.56±0.55 b**	**20.82±0.96 b**	**22.71±1.27 b**	18.72±0.99	1.08±0.07
T × Y							
Multi-stemmed	2007	**5848.9±2295.3**	**4.13±0.52**	**22.19±1.14**	**24.06±1.03**	**19.18±1.07**	1.08±0.06
	2008	**8758.0±2658.5**	**3.96±0.34**	**21.97±1.03**	**23.53±0.98**	**19.41±0.91**	1.07±0.07
	2014	n.a.	**3.40±0.46**	**20.74±0.88**	**22.36±1.04**	**18.75±0.98**	1.07±0.07
Single trunk	2007	**1732.3±345.01**	**3.52±0.47**	**20.71±0.93**	**23.51±1.09**	**18.19±0.78**	1.13±0.06
	2008	**3820.2±2010.2**	**3.68±0.55**	**20.59±1.24**	**23.62±1.44**	**18.27±1.17**	1.14±0.08
	2014	n.a.	**3.71±0.59**	**20.91±1.03**	**23.08±1.39**	**18.69±0.99**	1.10±0.08
Probability levels							
T		***	n.s.	***	n.s.	***	***
Y		***	***	***	***	n.s.	n.s.
T x Y		*	***	***	***	***	n.s.

sented with higher values in 2014, but similar to 2008, while lower values were recorded in fruits from 2007. Considering the interaction of the studied factors, a significant effect was only found for two of the kernel traits, namely its length and thickness.

Kernel phenolic content and antioxidant activity

There was a significant effect of the cultivar on the total phenolic content, resulting in higher amounts detected in 'Segorbe', compared to 'Butler' (Table 5), while no effect of the training system was found. Furthermore, the interaction be-

Table 2. Values (mean ± standard deviation) for yield and fruit characteristics of 'Segorbe' cultivar, and probability levels of the effects of Training System (multi-stemmed shrubs and free single trunk) and Year, as determined by two-way ANOVA. Probability levels: n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001. In bold, results that showed to be affected by the studied factors or their interaction.

		Yield (kg tree^{-1})	Fruit weight (g)	Fruit width (mm)	Fruit length (mm)	Fruit thickness (mm)	Fruit roundness index
Total		4564.6±1835.8	2.96±0.66	19.32±1.61	20.69±2.19	17.29±1.29	1.07±0.08
Training system (T)							
Multi-stemmed		**3787.9±1289.8 b**	**2.94±0.62 b**	19.39±1.63	**20.36±1.89 b**	17.34±1.33	**1.05±0.06 b**
Single trunk		**5341.2±2021.6 b**	**2.98±0.70 a**	19.25±1.59	**21.03±2.41 a**	17.23±1.25	**1.09±0.08 a**
Year (Y)							
2007		**3728.7± 906.9 b**	**3.16±0.57 b**	**20.04±1.22 b**	**21.63±2.21 b**	**17.49±1.13 b**	**1.07±0.09 a**
2008		**5261.1±2143.9 a**	**3.82±0.47 a**	**21.28±1.32 a**	**23.57±1.22 a**	**18.84±1.19 a**	**1.10±0.08 a**
2014		n.a.	**2.60±0.39 c**	**18.42±0.99 c**	**19.43±1.11 c**	**16.70±0.86 c**	**1.05±0.06 b**
T × Y							
Multi-stemmed	2007	2208.8±765.4	**2.79±0.41**	**19.37±1.12**	**19.74±1.16**	**16.79±0.98**	**1.01±0.06**
	2008	4270.5±1497.9	**3.96±0.34**	**21.97±1.03**	**23.53±0.98**	**19.41±0.91**	**1.07±0.07**
	2014	n.a.	**2.65±0.31**	**18.54±0.83**	**19.52±1.02**	**16.84±0.77**	**1.05±0.62**
Single trunk	2007	4248.6±767.7	**3.52±0.47**	**20.71±0.93**	**23.51±1.09**	**18.19±0.78**	**1.13±0.06**
	2008	6251.8±2348.1	**3.68±0.55**	**20.59±1.24**	**23.62±1.44**	**18.27±1.17**	**1.14±0.08**
	2014	n.a.	**2.56±0.46**	**18.31±1.13**	**19.34±1.19**	**16.56±0.92**	**1.05±0.07**
Probability levels							
T		*	*	n.s.	***	n.s.	***
Y		*	***	***	***	***	***
T x Y		n.s.	***	***	***	***	***

Table 3. Values (mean ± standard deviation) for kernel characteristics of 'Butler' cultivar, and probability levels of the effects of Training System (multi-stemmed shrubs and free single trunk) and Year, as determined by two-way ANOVA. Probability levels: n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001. In bold, results that showed to be affected by the studied factors or their interaction.

		Kernel/fruit weight	Kernel weight (g)	Kernel width (mm)	Kernel length (mm)	Kernel thickness (mm)	Kernel roundness index
Total		0.46±0.07	1.70±0.22	15.03±1.11	18.43±1.10	13.22±0.99	1.23±0.12
Training system (T)							
Multi-stemmed		**0.47±0.08 a**	1.70±0.19	15.18±1.07	**18.22±1.11 b**	13.43±0.94	**1.20±0.12 b**
Single trunk		**0.46±0.05 b**	1.70±0.25	14.88±1.12	**18.65±1.05 a**	13.00±0.99	**1.26±0.12 a**
Year (Y)							
2007		0.45±0.02 b	1.74±0.24	**14.74±1.29 a**	**19.21±0.97 a**	**12.86±0.91 b**	**1.31±0.14 a**
2008		0.45±0.03 b	1.72±0.21	**14.70±1.15 a**	**18.69±1.18 b**	**13.12±1.03 a,b**	**1.27±0.14 a**
2014		0.48±0.08 a	1.68±0.22	**15.23±0.97 b**	**18.08±0.95 c**	**13.37±0.97 a**	**1.19±0.09 b**
T × Y							
Multi-stemmed	2007	**0.45±0.02**	**1.86±0.19**	**15.24±1.17**	19.41±1.01	13.25±0.94	1.28±0.13
	2008	**0.44±0.03**	**1.78±0.19**	**15.03±1.12**	18.49±0.86	13.35±0.98	1.23±0.12
	2014	**0.48±0.09**	**1.62±0.14**	**15.20±1.03**	17.72±0.85	13.52±0.93	1.17±0.10
Single trunk	2007	**0.46±0.03**	**1.62±0.21**	**14.24±1.24**	19.01±0.90	12.46±0.70	1.34±0.14
	2008	**0.45±0.02**	**1.66±0.22**	**14.37±0.10**	18.89±1.41	12.89±1.04	1.32±0.15
	2014	**0.47±0.06**	**1.74±0.26**	**15.26±0.92**	18.45±0.92	13.22±0.99	1.21±0.08
Probability levels							
T		**	n.s.	n.s.	***	n.s.	***
Y		n.s.	n.s.	***	**	**	***
T × Y		***	**	**	n.s.	n.s.	n.s.

tween the cultivar and training system also had a significant influence on the total phenolic content. Indeed, for 'Butler', a higher amount of total phenolic was found when trained as multi-stemmed trees while for 'Segorbe', fruits from single-trunk trained trees presented a higher total phenolic content. When analyzing the data of the antioxidant assays, no clear influence pattern of the cultivar, training system or their interaction was expressed (Table 5). In fact, both the training systems and the interaction of factors had a significant effect on radical scavenging capacity, measured using DPPH assay, while for the FRAP assay, the influence was exerted by training system and cultivar, proving this latter factor to be the only one influencing the results for the ABTS assay. For the DPPH assay, the extremely significant effect of the training system was revealed by the higher antioxidant activity recorded for kernels of multi-stem trained plants. Similarly, the FRAP assay was also significantly influenced by the training system, with results showing a higher anti-

Table 4. Values (mean ± standard deviation) for kernel characteristics of 'Segorbe' cultivar, and probability levels of the effects of Training System (multi-stemmed shrubs and free single trunk) and Year, as determined by two-way ANOVA. Probability levels: n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001. In bold, results that showed to be affected by the studied factors or their interaction.

		Kernel/fruit weight	Kernel weight (g)	Kernel width (mm)	Kernel length (mm)	Kernel thickness (mm)	Kernel roundness index
Total		0.46±0.04	1.24±0.15	14.45±0.98	14.64±1.04	13.01±0.85	1.01±0.99
Training system (T)							
Multi-stemmed		0.46±0.04	1.25±0.14	14.51±0.89	14.68±0.95	13.12±0.82	1.01±0.09
Single trunk		0.46±0.04	1.23±0.17	14.39±1.06	14.59±1.12	12.89±0.87	1.01±0.11
Year (Y)							
2007		**0.45±0.02 b**	**1.29±0.15 a**	**15.01±0.97 a**	14.66±0.93	**13.19±0.80 a**	**0.97±0.09 b**
2008		**0.44±0.03 c**	**1.26±0.12 a,b**	**14.62±0.85 b**	14.56±1.07	**13.24±0.71 a**	**0.99±0.09 a,b**
2014		**0.47±0.04 a**	**1.23±0.16 b**	**14.21±0.94 c**	14.66±1.07	**12.87±0.88 b**	**1.03±0.99 a**
T × Y							
Multi-stemmed	2007	0.46±0.02	1.28±0.16	15.07±1.01	**14.96±0.94**	**13.02±0.88**	0.99±0.08
	2008	0.44±0.03	1.22±0.12	14.63±0.69	**14.34±0.88**	**13.34±0.75**	0.98±0.08
	2014	0.47±0.04	1.25±0.13	14.29±0.84	**14.70±0.96**	**13.08±0.82**	1.03±0.09
Single trunk	2007	0.45±0.03	1.29±0.14	14.96±0.95	**14.37±0.86**	**13.37±0.68**	0.96±0.09
	2008	0.43±0.03	1.25±0.13	14.62±0.99	**14.78±1.21**	**13.15±0.66**	1.01±0.11
	2014	0.47±0.05	1.20±0.19	14.12±1.03	**14.61±0.17**	**12.65±0.90**	1.03±0.10
Probability levels							
T		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Y		***	*	***	n.s.	**	***
T × Y		n.s.	n.s.	n.s.	*	**	n.s.

Table 5. Values (mean ± standard deviation) for kernel total phenolic content and antioxidant activity and probability levels of the effects of Cultivar ('Butler' and 'Segorbe') and Training System (multi-stemmed shrubs and free single trunk), as determined by two-way ANOVA. Probability levels: n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001. In bold, results that showed to be affected by the studied factors or their interaction.

	Total phenolics (mg gallic acid g^{-1})	DPPH (mmol Trolox kg^{-1})	FRAP (mmol Trolox kg^{-1})	ABTS (mmol Trolox kg^{-1})
Cultivar (C)				
'Butler'	**0.71±0.10**	2.87±0.74	**3.16±0.59**	**9.38±1.09**
'Segorbe'	**0.77±0.12**	2.88±0.91	**4.22±0.90**	**7.86±1.09**
Training system (T)				
Multi-stemmed	0.75±0.09	**3.21±0.69**	**3.91±0.95**	8.66±1.41
Single trunk	0.73±0.14	**2.55±0.82**	**3.48±0.87**	8.58±1.26
C × T				
'Butler' Multi-stemmed	**0.75±0.07**	**3.02±0.48**	3.27±0.34	9.41±1.15
'Butler' Single trunk	**0.66±0.12**	**2.72±0.92**	3.05±0.76	9.34±1.07
'Segorbe' Multi-stemmed	**0.74±0.11**	**3.39±0.83**	4.54±0.94	7.90±1.26
'Segorbe' Single trunk	**0.80±0.12**	**2.37±0.67**	3.91±0.76	7.82±0.95
Probability levels				
Cultivar (C)	*	n.s.	***	***
Training system (T)	n.s.	***	*	n.s.
C × T	**	*	n.s.	n.s.

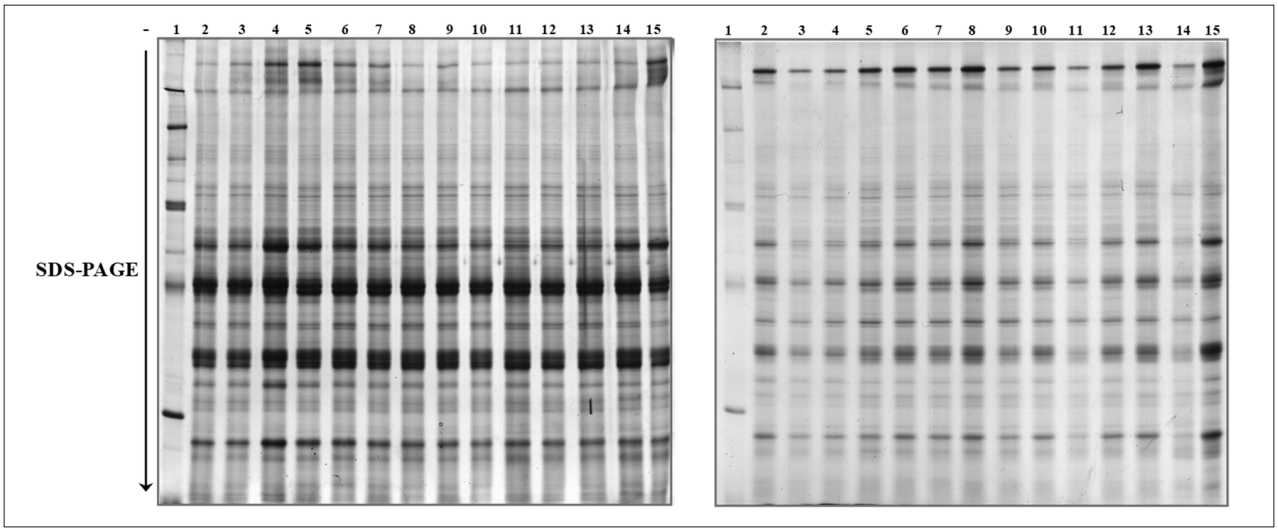


Figure 1. Representative pattern of high molecular weight (left) and low molecular weight (right) SDS-PAGE patterns obtain from the characterization of the hazelnut cultivars. Lane 1 (Molecular Marker: 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa and 250 kDa); Lane 2, 3, 4, 8, 9 and 10: 'Butler' Multi-stemmed; Lane 5, 6 and 7, 11, 12 and 13: 'Butler' Single trunk; Lane 14 and 15: 'Segorbe' Single trunk. Pattern for 'Segorbe' Multi-stemmed samples, and further repetitions of this sample samples were performed in additional SDS-PAGE (not presented) and showed the same pattern.

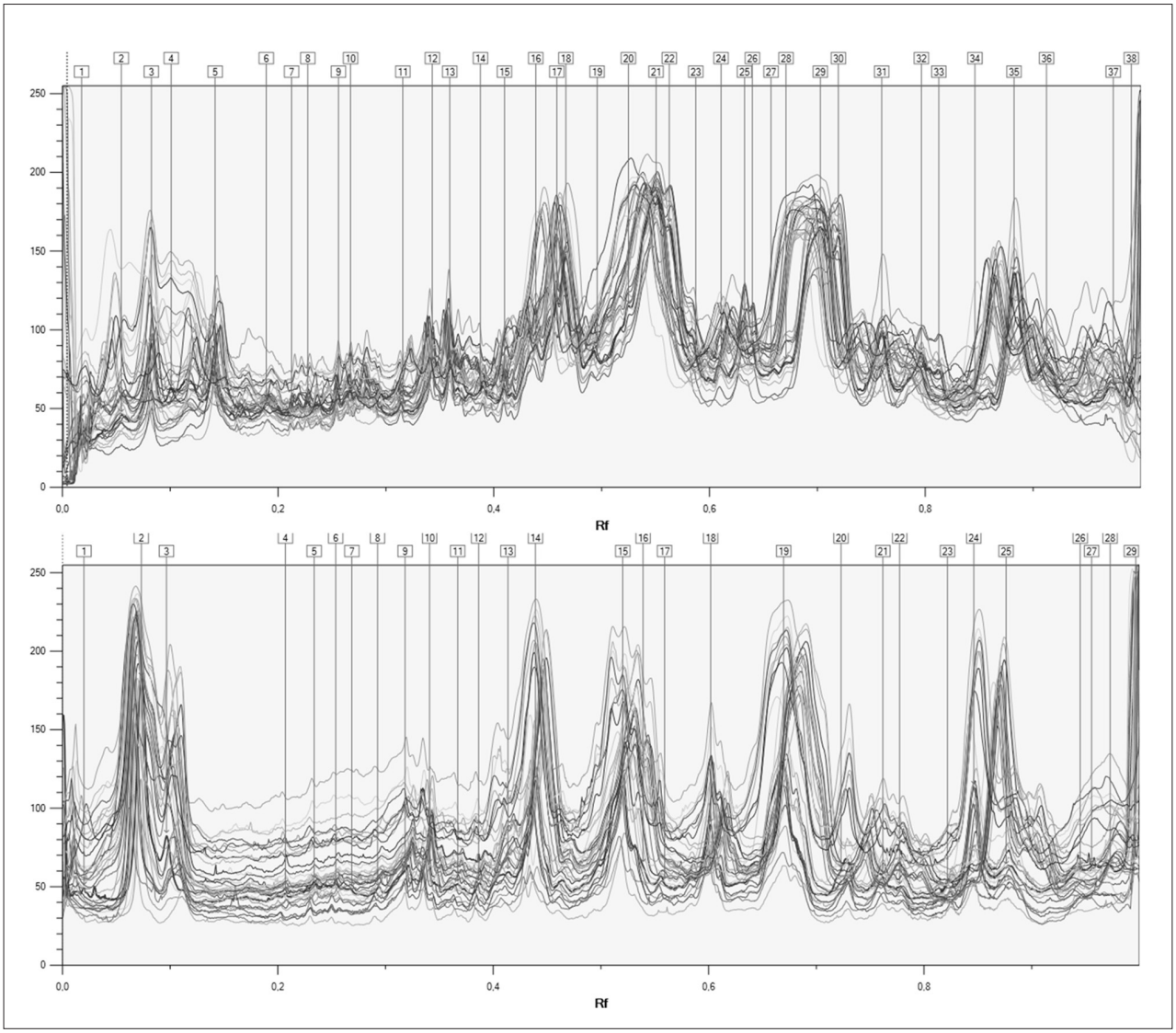


Figure 2. Phoretix program analysis of the genotypes according to their protein patterns. High molecular weight (up) and low molecular weight (down). Each number corresponds to a detected but not identified protein.

oxidant activity for multi-stem trained plants. Although this same influence of training system was not found for the ABTS assay, a pattern is visible, with higher values always found for multi-stemmed training, including for total phenolic content. Regarding the interaction of the two factors, a significant influence was detected on the DPPH assay, with higher values observed, in both cultivars, for multi-stemmed training.

SDS-PAGE profiles

The molecular diversity of the two hazelnut cultivars, growing under the two studied training systems, was analyzed by 1-DE resulting in two sets of gels for high and low molecular weight proteins (Figure 1). The comparison between cultivars/training systems was possible with Phoretix (Figure 2), by overlaying of protein profiles it generated spectra which facilitated the observation of presence/absence of bands. By analysis of the spectra of each hazelnut cultivar it was possible to observe a repetition of the protein profiles, in particular a repetition of number, position and intensity of the bands. Each band marked by the program corresponds to a peak in a generated spectrum made for comparison of protein profiles. A total of 38 bands corresponding to HMW globulins and 29 bands corresponding to LMW globulins were identified. No differences were found in the protein profiles, when comparing different cultivars and training systems under evaluation.

Discussion

Yield and fruit and kernel characteristics

Training systems are known to influence yield of hazelnut trees (Me et al., 2001; Silva et al., 2005) and this factor appears to be linked to the amount and location of the light interception occurring within the trees (Germaine and Sarraquigne, 1997). As we did not evaluate of light penetration into canopies, we cannot clearly link this fact to our results. Furthermore, the variation of the training system effect, depending on the used cultivar, was already reported (Me et al., 2001; Silva et al., 2005). Regarding fruit and kernel characteristics, differences between these traits observed in both studied cultivars, are well documented, and are a part of the parameters that differentiate each cultivar (Yao and Mehlenbacher, 2000; Farinelli et al., 2005; Gonçalves et al., 2009; Solar and Stampar, 2011). Variations of fruit weight, for the same cultivar, caused by an influence of the training system have been previously reported (Farinelli et al., 2005). In that work, an increase of the fruit and kernel weight was recorded, for 'Tonda di Giffoni' and 'Tonda Gentile Romana' cultivars, with a decrease of canopy density, which allows a better penetration of light within the tree. Furthermore, similar results were found for Barcelona and Ennis cultivars, where shading of the tree canopies results in a considerable fruit weight reduction (Gonçalves et al., 2009). However, our results show that for the studied cultivars, in these particular conditions, the different training systems have no influence on kernel weight, and the fruit weight was only influenced by the training system for 'Segorbe'. Data for comparison of fruits and kernels dimensions is very scarce, but, however, previous data indicate similar dimensions for 'Segorbe' fruits (Yao and Mehlenbacher, 2000) although smaller dimensions have been reported for fruits of both cultivars (Solar and Stampar, 2011). The training system also influenced fruit and kernel dimensions, which can be explained by the different light conditions in each system. In fact, the effect of light environments on fruit and kernel dimensions have been previously described (Farinelli et al., 2005), explaining that less dense canopies led to an increase of both width and length of the fruits and kernels of 'Tonda di Giffoni' and 'Tonda Gentile Romana' cultivars.

The variation of the yield and fruit and kernel traits between years is already known in hazelnut but does not show a clear pattern of influence, as it may vary from cultivar to cultivar (Beyhan, 2007; Solar and Stampar, 2011). Climatic conditions in the years of the study present some considerable difference, which may be responsible for these changes on yield and fruit characteristics (Bignami et al., 2000). The year of 2007 was the second-driest year recorded in Portugal since 1931; 2008 was the 8th driest, while 2014 recorded increased average precipitation values (IPMA, 2017). On the other hand, average air temperature recorded in 2014 was the 11th highest since 1931, while values for the years 2007 and 2008 were average considering the air temperature. Interestingly, overall results, in contrary to what has been found for other fruit crops (e.g., Faust, 1989) and for hazelnut (Silva et al., 2003), higher nut and/or kernel weight were not found when productivity was lower, as it would be expected due to less partitioning of photoassimilates, as higher source/sink rates occur. In our work, this was only recorded for 'Butler' in the multi-stemmed training, while in the single trunk training, and for both training systems in 'Segorbe', this behaviour was not found. Regarding the RI, similar values have been previously described for both 'Butler' and 'Segorbe' fruits and kernel (Beyhan, 2007) indicating a rounder shape for the kernel of 'Segorbe', which is further confirmed by other shape indexes (Solar and Stampar, 2011). Other authors have shown the effect of the light intensity, due to canopy density on shape indexes, which can help to explain the influence of training systems detected in this parameter. Farinelli et al. (2005) observed that a decrease of canopy density led to a reduction of the fruits' RI, while, on the other hand, the kernels presented higher RI. The same work showed that this behaviour is considerably related to the analyzed cultivar, and cannot be assumed to take place in every situation. Obviously, these changes on RI are linked to the variations caused by fruit and kernel dimensions used to calculate RI.

Kernel phenolic content and antioxidant activity

Regarding the total phenolic content, and using the same extraction methodology, Ghirardello et al. (2013) quantified 1.40 ± 0.29 g gallic acid kg^{-1}, a considerably higher amount, in kernels of 'Tonda Gentile delle Langhe'. This higher value, comparing to the ones observed in the present work, is related to the tested cultivar, as variation of total phenolic content between cultivars has already been reported in several previous works (Oliveira et al., 2008; Solar et al., 2009; Jakopic et al., 2011). Furthermore, a higher phenolic content of 'Segorbe' kernels, comparing to 'Butler', was also found (Solar and Stampar, 2011) although the inverse situation has also been reported, with high phenolics amount in 'Butler' kernels (Solar et al., 2009). These discrepancies can also be explained by the use of different extraction methodologies, which could lead to different values of total phenolics (Alasalvar et al., 2006; Delgado et al., 2010). The significant differences of antioxidant capacity observed between the cultivars have also been already reported in several works (Oliveira et al., 2008; Jakopic et al., 2011; Locatelli et al., 2011). Those previous results available also indicate variations of the antioxidant capacity, depending on the extraction and antioxidant assay methodology, but, similar results have already been reported. Arcan and Yemenicioğlu (2009) report between 12.8 to

17.83 mmol Trolox kg^{-1} of dry weight, depending whether water or ethanol was used as extraction solvent, in the ABTS assay. Although no previous data were found regarding the influence of the training system on the antioxidant activity of the hazelnut kernels, their influence on leaf characteristics is well established, and those can ultimately lead to variation on the chemical compositions of fruits, which can subsequently influence their antioxidant capacity. Moreover, the antioxidant capacity of hazelnut can be linked to the content of phenolic compounds. Indeed, several works point out the antioxidant activity of phenolic compounds (Vermerris, 2007) and our results also showed that the phenolic content was correlated in each of the training systems to the antioxidant activity studied by the DPPH (for multi-stemmed training, P = 0.01, 0.759 and for single trunk, P = 0.01, 0.545) and FRAP (for multi-stemmed training, P = 0.01, 0.525, and for single trunk, P = 0.01, 0.900). In the present work, 'Segorbe' presented higher antioxidant activity in the FRAP assay (4.22 ± 0.15 mmol Trolox kg^{-1}), while, for ABTS, better results were achieved using kernels from 'Butler' (9.38 ± 0.18 mmol Trolox kg^{-1}). The results of the FRAP and ABTS assays were correlated to the phenolic content in each cultivar (P = 0.01, 0.626 and P = 0.01, 0.644 for 'Segorbe' and FRAP and ABTS, respectively; in 'Butler', P = 0.01, 0.873 and P = 0.01, 0.743 for FRAP and ABTS, respectively). However, others works point out that different chemical reactions are behind the antioxidant assays (Huang et al., 2005). They furthermore report that chemical composition of the samples can influence the results, regardless of the presence of phenolics. This may help to understand the results of DPPH and ABTS assays, which don't follow the same trend presented by the phenolic contents.

SDS-PAGE patterns

Based on the obtained results, the levels of inter- and intra-cultivar variability in the analysed germplasm were relatively reduced, since there is a group of profiles that appear in a repetitive way within the same cultivar, but also in different cultivars and training systems. European hazelnut is a diploid with 11 chromosomes ($2n=2x=22$) and its genome is relatively small (~378 Mbp). The reduced level of inter- and intra-genetic diversity directly involves a low intraspecific genetic diversity based on the study of storage proteins by the technique of SDS-PAGE. By using SDS-PAGE and silver staining, it was previously shown that the protein patterns of different hazelnut varieties are quite similar (Mueller et al., 2002). Homology-based functional annotation predicted 34,910 protein coding loci, with 22,474 having homology to an entry in the NCBI non-redundant protein database. This result comes to note the lower intra- and inter-variability present among genotypes/varieties and the importance of continuing the analysis of genetic diversity in this specie but with higher resolution techniques. In fact hazelnuts are a common cause of food allergy (Nagaraju et al., 2016) and are used increasingly in the United States and Europe. Through the use of SDS-PAGE, 2-dimensional (2-D) proteomics, mass spectrometry, and internal sequencing these genotypes could be evaluated in order to identify new allergens responsible for these reactions.

Conclusions

Our results show significant influence that the training system has on some morphological and biochemical parameters of fruits and kernels of 'Butler' and 'Segorbe' cultivars. Regarding 'Butler' cultivar, it appears that from the production point of view, the multi-stemmed training is to be recommended rather than the single trunk. In fact, for this cultivar, this training system appears to be the one to select, as higher yields and larger fruits were observed. In the 'Segorbe' cultivar, production data indicates a difference between training systems, with higher production recorded in the single trunk training, as well as higher fruit weight. These data should be taken into consideration when planting new hazelnut orchards. Future work is necessary to obtain the full sequence of proteins profiles identified by SDS-PAGE in order to verify their role as seed allergens.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Addresses of authors:
Ivo Oliveira[1,*], Ana Paula Silva[1], Tiago Santos[2],
Gilberto Igrejas[2,3] and Berta Gonçalves[1]
[1] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[2] Functional Genomics and Proteomics Unit, Department of Genetic and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[3] UCIBIO-REQUIMTE, Faculty of Science and Technology, New University of Lisbon, Monte da Caparica, Portugal
* Corresponding author; E-mail: ivo.vaz.oliveira@utad.pt