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Leaf age, seasonal and annual variations in *Salvia officinalis* L. var. *purpurascens* biochemical characteristics

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Summary

Medicinal and aromatic plants (MAP's) have gain new attention in the past years due to their content in bioactive compounds and recognized health-promoting effects. One of the most important species of MAP's is *Salvia officinalis* L., rich in several phytochemicals (essential oil, phenolic compounds) and vitamins. Besides, it has various uses and pharmacological effects (including antibacterial, antiviral, antioxidative, anti-inflammatory, antidiabetic and antitumour activities). *Salvia officinalis* L. has many cultivars, including *Salvia officinalis* L. var. *purpurascens*, which is currently understudied. As few is known about this specific cultivar, characterization of this plant, as well as the study of biochemical variations occurring during its development, is of great significance. Hence, in this work, young and adult leaves of *Salvia officinalis* L. var. *purpurascens*, were collected in two different seasons (June and September) and in two different years (2011 and 2013). Several biochemical traits were analyzed, namely carbohydrate content, photosynthetic pigments concentration, total phenolics, soluble proteins, as well as oxidation parameters (thiobarbituric acid reactive substances, thiols and electrolyte leakage). The Year factor significantly influenced carbohydrate content (higher in 2013 for non-structural carbohydrates and soluble sugars, but lower for starch), but also chlorophyll and carotenoid content (higher in 2011), with a similar influence recorded for the Season of harvest (higher values for starch, chlorophyll and carotenoids in September, but lower for soluble sugars). The developmental stage of leaves showed significant influence mainly in the content of photosynthetic pigments, with higher values of chlorophyll and carotenoids recorded in young leaves. The results show the biochemical variations occurring in plants of *Salvia officinalis* L. var. *purpurascens*, during developmental stages, and others associated to season of harvest and year, and their relation to climatic factors. The gathered data, besides useful for the characterization of this plant, is also valuable when aiming for the optimization of sage cultivation.

Keywords
Salvia officinalis L. var. *purpurascens*; carbohydrates; chlorophyll, carotenoids; total phenolics

Introduction

In recent years, there has been an increased interest in herbs and spices, due to their documented and studied beneficial health effects. These plants have in their composition a large number of bioactive and health-promoting compounds, including vitamins, terpenoids and polyphenols, with several studies showing their beneficial effects in cancer, cardiovascular disease and neurodegenerative disorders (Halliwell, 1996). One of the most studied aromatic plants is *Salvia officinalis* L. – common sage – native to the Mediterranean region and Asia Minor, which has been used in folk medicine for centuries, it is known to have pharmacological effect, which resulted in its inclusion in pharmacopoeias throughout the world (Tepe, 2008). Together with the increased demand of new sources of bioactive compounds, based in a global trade of herb-based products that reached an estimated amount S$ 60 000 million in 2000 (World Health Organization, 2003), renovated interest in sage has been triggered. Due to its economic value, this plant has been included in cultivation systems, which can help to achieve higher plant quality (Schippmann et al., 2006). However, sage is a drought-susceptible species (Tounekti et al., 2011), and in the Mediterranean areas, this plant in under summer drought stress, together with high temperatures, which can lead to variations on several characteristics. Some available works regarding *Salvia officinalis* L. focused on the variations of growth parameters, volatile composition, essential oils and phenolics, caused by the type of cultivation (field or greenhouse) (Yi and Wetzstein, 2010), saline stress (Taârit et al., 2012), low light conditions (Mapes and Xu, 2014) or water deficit (Bettaieb et al., 2011) rather than in biochemical parameters of this plant. Furthermore, *Salvia officinalis* L. has many known varieties, being *Salvia officinalis* var. *purpurascens* one of the less studied ones. Hence, it is of essential interest to study the biochemical variations occurring in sage leaves that can be of great importance to achieve maximum quality, from the producers' standpoint, but also from consumer's point of view. Therefore, in this work we present results from biochemical characteristics, and their variation caused by leaf age, season or year in *Salvia officinalis* var. *purpurascens*.

Materials and methods

Plant material

Salvia officinalis L. var. *purpurascens* plants were collected from the Botanical Garden of the University of Trás-os-Montes e Alto Douro (UTAD), Vila Real (41º19' N, 7º44' W, 450 m above sea level), having the herbarium specimen number "HVR13737, Gerês, 05-11-2007, J.M. Neves". Plants are on a dystric cambisol (Non-humic litholic) derived from shale. Is presents a medium texture (fine-sandy) with acidic pH (5.4), a percentage of organic matter of 1.45 and average phosphorus (63 ppm) and very high potassium (348 ppm) contents. No fertilization is applied, and watering of plants is performed regularly. Climatic data was recorded by a standard weather station located near the experimental site. The study was carried out in 2011, 2012 and 2013, but, considering the differences regarding several climatic conditions (namely precipitation, temperature and total radiation) recorded for 2011 and 2013 (Fig. 1) (while conditions were similar between 2012 and 2013), we choose to present result concerning these two years. Healthy leaves, presenting two developmental stages (young, collected from the upper part of the plant and adult, collected from the middle third), were collected at two different harvest dates (June and September) and

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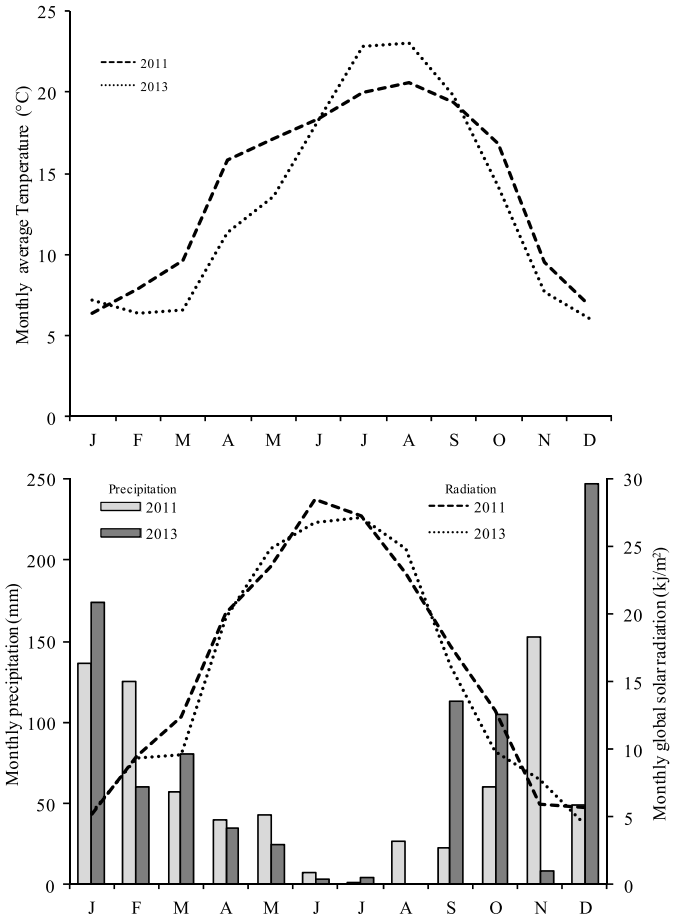

Fig. 1: Average monthly temperature (°C), total monthly precipitation (mm) and global solar radiation (kJ/m^2) for 2011 and 2013.

years (2011 and 2013). Samples were obtained from twelve 4 years-old plants and eight repetitions of all methodologies were performed in randomly selected leaves. Leaf discs were prepared in the field, deep-frozen in liquid nitrogen and stored at -80 ºC until analysis.

Carbohydrate content

Carbohydrate content was measured using the methodology of IRIGOYEN et al. (1992), by heating (80 °C) one leaf disc in 80% (v/v) ethanol/distilled water solution, for 1 hour. Afterwards, 0.2 mL of the previous extract and 3 mL of anthrone were mixed and placed in a water bath at 100 ºC, during 10 minutes. The liquid fraction was used for soluble sugar quantifications and the solid fraction was used for starch analysis. The solid fraction was extracted with 30% perchloric acid and quantified according to OSAKI et al. (1991), following the anthrone procedure described in the soluble sugars methodology, using glucose as standard in both methodologies.

Photosynthetic pigments

The quantification of chlorophyll (Cla and Clb) and total carotenoids was performed using the methods of SESTÁK et al. (1971) and LICHTENTHALER (1987), respectively, by spectrophotometry in extracts of 80% acetone with distilled water (v/v).

Total phenolics

A modified procedure of the Folin-Ciocalteu method (TSAO et al., 2003) was used for accessing the concentration of phenolic compounds, using the same extracts obtained for the quantification of photosynthetic pigments. Results are expressed as mg of gallic acid equivalents, using a calibration curve obtained by preparing different known concentrations of gallic acid, measured using the same procedure.

Soluble proteins

Soluble proteins were quantified by homogenising samples in a grinding medium containing 50 mM phosphate buffer (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (w/v) polyvinylpyrollidone (PVP), followed by centrifugation at 22 000 g for 30 minutes, at 4 ºC. Absorbance was read at 595 nm, and bovine serum albumin (BSA) was used as a standard (BRADFORD, 1976).

Thiobarbituric acid reactive substances

The concentration of total thiobarbituric acid reactive substances (TBARS) was calculated to evaluate membrane integrity. Lipid peroxidation in *Salvia officinalis* var. *purpurascens* leaves was estimated following the method described in BACELAR et al. (2006). Briefly, samples previously frozen with liquid nitrogen were ground in 3 mL of 20% (w/v) trichloroacetic acid (TCA) using mortar and pestle, homogenized and centrifuged at 3500 g for 20 min. Afterwards, 1 mL of the supernatant was added to 1 mL of 20% TCA containing 0.5% (w/v) thiobarbituric acid and to 100 µL 4% (w/v) butylated hydroxytoluene (BHT). This mixture was heated at 95 ºC for 30 min, quickly cooled in an ice bath and centrifuged at 10,000 g for 20 min and the absorbance of the supernatant was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The TBARS concentration was expressed in terms of nmol/g and nmol/cm^2, using an extinction coefficient of 155,000 M^{-1} cm^{-1}.

Thiols

Total thiol content (–SH) of soluble protein extract was assayed as described in BACELAR et al. (2006), using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB).

Electrolyte leakage

Electrolyte leakage was measured following the methodology of LUTTS et al. (1996). Leaf discs were washed with deionised water to remove surface-adhered electrolytes, placed in closed vials containing 10 mL of deionised water and incubated at 25 ºC on a rotary shaker for 24 h. Afterwards, electrical conductivity (EC_1) of the solution was determined. Samples were then autoclaved at 120 ºC for 20 min and the electrical conductivity (EC_2) was obtained after equilibration at 25 ºC. The electrolyte leakage was calculated as follows: Electrolyte leakage = $\left(\frac{EC_1}{EC_2}\right) \times 100$.

Statistical analysis

Data are presented as mean ± standard deviation, and the results presented by weight refer to dry weight of sage leaves. 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.) software. The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro-Wilk's test (if $n < 50$), and the Levene's tests, respectively.

Results and discussion

There are few previous works regarding the analyzed biochemical parameters of *Salvia officinalis* var. *purpurascens,* thus, most of the comparisons here presented are made with *Salvia officinalis* related works (rather than with var. *purpurascens*), unless stated otherwise. The sampling Year proved to be the factor that significantly influenced a higher number of parameters (Tab. 1, 2 and 3). Considering all the results, sixteen of the twenty-five analyzed biochemical parameters were affected by the Year of study. Of those, non-structural carbohydrates, soluble sugars, soluble sugars/starch ratio, total chlorophyll/total carotenoids ratio, total phenolics and electrolytes leakage presented higher values in 2013, while starch, chlorophyll a (expressed as mg/g), total chlorophyll (expressed as mg/g), total carotenoids, and TBARS (expressed as nmol/g) showed higher values in 2011. Seasonal variations of the studied biochemical parameters were also detected (Tab. 1, 2 and 3). Of those significantly affected by the sampling date, starch, chlorophyll a, total chlorophyll (expressed as mg/g) and total carotenoids were present in higher amounts in leaves collected in September, while soluble sugars, soluble sugars/starch, total chlorophyll (expressed as mg/dm^2) and thiols were detected in higher concentrations when leaves were collected in June. Finally, the leaf developmental stage caused fewer significant variations in biochemical parameters of sage. The leaf age (young or adult) only significantly affected ten out of 25 parameters. Of those, only sugars/starch ratio was higher in adult leaves, while content of chlorophyll a, chlorophyll b (expressed as mg/dm^2), total chlorophyll, total carotenoids and TBARS was higher in young leaves.

Few works are available regarding carbohydrate (soluble sugars and/or starch) content of *Salvia officinalis*. Sahar and colleagues (2011) recorded contents for soluble sugars of about 9 mg/g of fresh leaf, while Castrillo et al. (2005) refer about 1.25 g/m^2. When reporting our data in fresh weight (the leaves tested in this work had an average of 73% of water content – data not shown), a content of about 50 mg/g of dry weight will correspond to about 13.5 mg/g fresh weight, similar to the reported 9 mg/g of Sahar et al. (2011). The values of 151.34±8.67 mg/g will represent over 40 mg/g expressed in fresh weight, considerably higher than those previous reports indicate. On the other hand, the values reported by Castrillo et al. (2005) (1.25 g/m^2, corresponding to 12.5 mg/dm^2) can also be considered similar to the ones detected in the present work (ranging from 4.57±0.31 mg/dm^2 in leaves from 2011 to 15.36±0.76 in leaves from 2013). The concentration of soluble sugars has been correlated to specific leaf mass, as well as with irradiance, but also to water stress, as they are known osmoprotectants and carbon sources (Castrillo et al., 2005). Total carbohydrate content of sage has been reported to be 15 μg/mg fresh weight (Pellegrini et al., 2015), well below the content detected by us, of around 54 μg/mg fresh weight (again, if considering 73% of water content). No reports concerning the starch content of sage are known. However, starch content in other aromatic and medicinal plants has been reported to present seasonal changes, increasing through spring until autumn (Kofidis et al., 2007). The lower value of starch in June, combined with higher values of soluble sugars, with an inverse behaviour in September, can be related to the mobilization of starch and its conversion in soluble sugars, as detected in other species. This will take place in order to support the increased metabolism during the flowering period of sage, which occurs between April and July, but always depending of climatic conditions. As sage is an important aromatic plant, effects of the composition on processing parameters (including cutting, drying or freezing) is of interest. No data regarding how carbohydrate content of sage can influence or be influenced by processing parameters is available. Nonetheless, reports concerning the effects of drying methodologies in the sugar content of several plants show considerable variations, depending on the selected process (e.g. Gao et al., 2012).

Concerning the chlorophyll content found in leaves of sage in the present work, similar amounts have been previously reported (Castrillo et al., 2005; Bettaieb et al., 2011; Tounekti et al., 2012). However, many reports also indicate higher (Mapes and Xu, 2014; Pellegrini et al., 2015) or lower values (Nasta et al., 2014). Several factors affecting the content of chlorophyll in sage, including ozone stress, light conditions, salinity, drought and temperature, can help to explain some of the variations detected between 2011 and 2013. Higher values for chlorophyll June, with a decrease in September can be related to the flowering period of sage. In fact, the work of Coisin et al. (2010), with *Salvia nemorosa*, recorded an increase in chlorophyll content when plants are in the anthesis stage, probably due to increased sugar synthesis, to support the metabolism of the plant. Furthermore, values of precipitation show a considerable difference between the amount of water that will be available for plants (average of both years for June is 5.6 mm, and is September of 67.8 mm). Considering that *Salvia officinalis* is a drought-susceptible species (Tounekti et al., 2011) this factor can also help to explain the recorded variations on chlorophyll content between June and September. The beginning of leaf senescence can be the cause for the decrease of chlorophyll content in adult leaves. In fact, during the senescence process, were reserves are mobilized for younger tissues, like developing leaves or flowers (Abreu and Munné-Bosch, 2007), chloroplasts are one of the first organelles to be subjected to senescence (Dangl et al., 2000). Significant variations were also detected in total carotenoids, correlated to all the factors under study (age, season and year). Although similar values have been previously published (Tounekti et al., 2012), other authors point out lower (Tounekti et al., 2011) or higher content (Mapes and Xu, 2014). Several factors are known to influence carotenoid content in sage, like ozone (Pellegrini et al., 2015), salinity (Tounekti et al., 2012) or light levels (Mapes and Xu, 2014). Our results show significant differences between young and adult leaves in the total carotenoid content, as detected for the chlorophyll content, which may be due to the onset of senescence that leads to losses of the compounds (Abreu and Munné-Bosch, 2007). Furthermore, differences in total carotenoid content between seasons and years are likely related to climatic factors, known to influence carotenoid content (Munné-Bosch and Alegre 2002), as they protect cellular structures by dissipating excess energy reaching the chloroplast, and by preventing the formation and/or scavenge any singlet oxygen that can result in lipid peroxidation in photosynthetic membranes. In fact, it was in 2011 that higher carotenoids and total radiation were recorded, results that may indicate a response to avoid oxidative damage caused by excess radiation. However, this same rationale cannot be used for results regarding seasonal variation. In fact, total radiation was lower in September; contrarily to what was found to carotenoid content (higher in September), indicating that other stress-causing factors, namely low rainfall, that occurred in June, may be affecting carotenoids content. No data was found regarding the correlation between processing of sage and its content in chlorophylls and carotenoids. However, data concerning other aromatic plants indicate a considerable reduction of the content of those compounds with processing (Divya et al., 2012). It can be expected that, during processing, leaves containing higher levels of carotenoids are less prone to suffer oxidative processes, as these compounds are known to have strong antioxidant activity (e. g. Krinsky, 1989).

For total phenolic content (Tab. 3), the recorded values can be considered similar to those found in previous works (e.g. Taârit et al., 2012; Yi and Wetzstein, 2012). However, other reports are available (Roby et al., 2013) that obtained different values of phenolic content in sage. These may be linked to several factors, such as growing conditions, but also to different approaches in the quantification of these compounds. For phenolics, interaction between

Tab. 1: Values (mean ± standard deviation) for carbohydrate content of *Salvia officinalis* leaves and probability levels of the effects of Age, Season and Year, as determined by ANOVA. ns: not significant. In bold, results that showed to be affected by the studied factors and/or their interaction.

	Non-structural carbohydrates (mg/dm^2)	Non-structural carbohydrates (mg/g)	Starch (mg/dm^2)	Starch (mg/g)	Soluble sugars (mg/dm^2)	Soluble sugars (mg/g)	Soluble sugars/ Starch
Age (A)							
Young	194.91±68.16	196.92±68.15	94.96±63.60	99.64±75.04	99.95±55.63	97.28±47.62	**3.06±4.33**
Old	202.30±80.35	213.69±80.35	102.95±80.93	109.27±83.19	99.35±71.19	104.43±75.93	**6.02±10.03**
Season (S)							
June	189.97±75.64	193.98±58.36	**72.26±78.50**	**76.19±85.11**	**117.61±72.23**	**117.79±74.41**	**8.36±9.67**
September	207.34±86.99	216.63±86.99	**125.65±54.71**	**132.73±60.89**	**81.68±47.74**	**83.91±43.92**	**0.71±0.49**
Year (Y)							
2011	**163.45±67.14**	**184.92±45.59**	**117.72±60.97**	**134.55±78.41**	**45.73±17.56**	**50.37±19.82**	**0.48±0.29**
2013	**233.76±63.79**	**225.70±67.34**	**80.19±78.64**	**74.36±67.69**	**153.57±43.03**	**151.34±49.04**	**8.59±9.49**
A*S							
Young*June	**164.71±46.24**	**149.49±46.24**	**47.02±36.94**	**41.47±28.61**	117.68±64.91	108.02±60.65	**5.52±5.08**
Young*September	**225.12±74.27**	**244.35±74.27**	**142.90±45.75**	**157.82±60.00**	82.21±38.88	86.53±27.62	**0.59±0.26**
Old*June	**215.04±56.57**	**238.48±59.57**	**97.49±100.07**	**110.90±107.62**	117.55±81.05	127.58±86.97	**11.21±12.26**
Old*September	**189.56±58.36**	**188.92±97.19**	**108.41±58.81**	**107.64±52.13**	81.15±56.56	81.28±56.64	**0.82±0.63**
A*Y							
Young*2011	157.49±45.59	180.55±45.59	103.59±42.87	121.29±78.88	**53.89±15.75**	**59.26±18.83**	**0.62±0.30**
Young*2013	232.33±67.34	213.29±67.34	86.33±79.77	77.99±66.46	**146.00±40.29**	**135.29±35.35**	**5.49±5.09**
Old*2011	169.39±84.63	189.29±84.63	131.85±73.61	147.82±78.16	**37.55±15.71**	**41.47±16.99**	**0.35±0.21**
Old*2013	235.20±80.35	238.11±62.21	74.06±79.61	70.72±70.90	**161.14±45.62**	**167.39±56.31**	**11.68±11.82**
S*Y							
June*2011	**182.32±75.64**	**190.03±75.54**	**130.87±73.33**	**139.06±80.72**	**51.45±18.15**	**50.97±9.09**	**0.54±0.36**
June*2013	**197.42±35.56**	**197.93±34.56**	**13.65±5.49**	**13.31±4.63**	**183.77±33.39**	**184.63±4.85**	**16.19±7.91**
September*2011	**144.56±53.28**	**179.80±53.24**	**104.57±43.98**	**130.04±78.40**	**39.99±15.42**	**49.76±26.99**	**0.43±0.21**
September*2013	**270.11±66.31**	**253.46±66.31**	**146.65±57.47**	**135.41±38.72**	**123.37±27.68**	**118.06±27.74**	**0.98±0.53**
A*S*Y							
Young*June*2011	**134.88±42.63**	**118.26±42.62**	**76.24±31.01**	**66.42±17.94**	58.64±19.81	51.83±10.56	**0.83±0.27**
Young*June*2013	**194.52±27.06**	**180.73±27.06**	**17.80±3.16**	**16.52±2.93**	176.72±25.85	164.21±23.52	**10.20±2.25**
Young*September*2011	**180.10±38.33**	**242.85±38.33**	**130.94±35.59**	**176.16±78.29**	49.15±9.37	66.69±22.84	**0.40±0.14**
Young*September*2013	**270.14±75.62**	**245.74±75.62**	**154.86±53.77**	**139.47±28.58**	115.28±25.55	106.37±14.59	**0.79±0.19**
Old*June*2011	**229.76±72.80**	**261.81±72.80**	**185.50±61.16**	**211.70±39.75**	44.26±13.98	50.11±7.92	**0.24±0.05**
Old*June*2013	**200.32±42.52**	**215.14±42.52**	**9.49±3.88**	**10.10±3.72**	190.83±40.19	205.04±49.29	**22.18±6.86**
Old*September*2011	**109.03±41.45**	**116.76±41.45**	**78.19±35.89**	**83.93±46.71**	30.84±15.18	32.83±19.62	**0.46±0.26**
Old*September*2013	**270.11±66.31**	**261.08±60.86**	**138.62±63.53**	**131.34±48.56**	131.46±28.96	129.74±33.53	**1.18±0.69**
F values and probability levels							
Age (A)	0.314n.s.	1.762n.s.	0.575n.s.	0.884n.s.	0.010n.s.	1.212n.s.	**21.232*** **
Season (S)	1.753n.s.	3.211n.s.	**25.678*** **	**30.514*** **	**35.357*** **	**27.222*** **	**142.081*** **
Year (Y)	**28.428*** **	**10.411** **	**12.685** **	**34.584*** **	**318.585*** **	**241.630*** **	**159.238*** **
A*S	**10.604** **	**32.637*** **	**16.258*** **	**34.138*** **	0.006n.s.	3.643n.s.	**18.146*** **
A*Y	0.117n.s.	0.405n.s.	3.689n.s.	2.725n.s	**6.790***	**14.742*** **	**25.235*** **
S*Y	**17.529*** **	**6.767***	**57.211*** **	**41.022*** **	**16.408*** **	**25.310*** **	**138.255*** **
A*S*Y	**9.208** **	**24.547*** **	**13.367*** **	**33.169*** **	0.062n.s.	0.319n.s.	**22.700*** **

Tab. 2: Values (mean ± standard deviation) for photosynthetic pigments of *Salvia officinalis* leaves and probability levels of the effects of Age, Season and Year, as determined by ANOVA. ns: not significant. In bold, results that showed to be affected by the studied factors and/or their interaction.

	Chlorophyll a (mg/dm^2)	Chlorophyll a (mg/g)	Chlorophyll b (mg/dm^2)	Chlorophyll b (mg/g)	Total chlorophyll (mg/dm^2)	Total chlorophyll (mg/g)	Cla/Clb	Total carotenoids (mg/dm^2)	Total carotenoids (mg/g)	Total chlorophyll /Carotenoids
Age (A)										
Young	**3.54±0.63**	**3.73±1.53**	**1.46±0.59**	1.55±0.85	**4.99±1.18**	**5.29±2.29**	2.56±0.42	**1.07±0.20**	**1.12±0.43**	4.72±1.05
Old	**2.89±0.63**	**3.10±0.79**	**1.17±0.44**	1.26±0.51	**4.06±0.96**	**4.36±1.19**	2.62±0.51	**0.87±0.12**	**0.94±0.19**	4.66±0.83
Season (S)										
June	**2.95±0.66**	**3.00±0.71**	1.25±0.56	1.28±0.61	**4.19±1.12**	**4.28±1.22**	2.53±0.51	**0.94±0.16**	**0.95±0.15**	4.53±1.12
September	**3.48±0.68**	**3.83±1.52**	1.38±0.50	1.54±0.78	**4.86±1.13**	**5.37±2.24**	2.64±0.42	**1.01±0.22**	**1.11±0.45**	4.84±0.69
Year (Y)										
2011	3.33±0.75	**3.82±1.55**	1.32±0.52	1.51±0.78	4.65±1.24	**5.33±2.27**	2.66±0.39	**1.05±0.21**	**1.18±0.42**	**4.44±0.82**
2013	3.09±0.67	**3.02±0.67**	1.31±0.55	1.30±0.62	4.41±1.09	**4.32±1.19**	2.51±0.52	**0.89±0.14**	**0.87±0.13**	**4.92±0.99**
A*S										
Young*June	3.33±0.64	3.10±0.79	1.39±0.69	1.31±0.75	4.72±1.28	4.41±1.49	2.55±0.43	1.04±0.14	**0.96±0.14**	4.60±1.35
Young*September	3.74±0.63	4.37±1.84	1.53±0.48	1.79±0.89	5.27±1.04	6.16±2.64	2.56±0.44	1.10±0.25	**1.28±0.56**	4.83±0.63
Old*June	2.57±0.42	2.90±0.63	1.09±0.37	1.24±0.46	3.67±0.59	4.14±0.89	2.52±0.59	0.83±0.09	**0.94±0.17**	4.46±0.86
Old*September	3.21±0.65	3.29±0.91	1.24±0.49	1.28±0.56	4.46±1.11	4.57±1.42	2.72±0.40	0.92±0.14	**0.94±0.22**	4.84±0.78
A*Y										
Young*2011	3.63±0.73	4.25±1.95	1.48±0.50	1.73±0.92	5.11±1.17	5.98±2.79	2.55±0.37	1.17±0.21	**1.34±0.52**	4.37±0.74
Young*2013	3.44±0.58	3.22±0.69	1.45±0.68	1.38±0.75	4.88±1.22	4.59±1.43	2.56±0.49	0.97±0.14	**0.90±0.10**	5.06±1.21
Old*2011	3.03±0.67	3.39±0.88	1.16±0.52	1.29±0.56	4.19±1.65	4.68±1.40	2.76±0.41	0.92±0.13	**1.03±0.19**	4.52±0.92
Old*2013	2.75±0.57	2.81±0.59	1.17±0.35	1.22±0.46	3.93±0.72	4.04±0.86	2.47±0.57	0.82±0.09	**0.84±0.14**	4.79±0.74
S*Y										
June*2011	**2.79±0.39**	**2.94±0.69**	**1.01±0.19**	**1.06±0.28**	**3.81±0.58**	**4.00±0.97**	2.79±0.20	**0.97±0.17**	**1.00±0.17**	3.97±0.39
June*2013	**3.11±0.83**	**3.06±0.74**	**1.48±0.71**	**1.49±0.77**	**4.59±1.39**	**4.55±1.40**	2.28±0.60	**0.90±0.14**	**0.89±0.11**	5.09±1.32
September*2011	**3.87±0.64**	**4.69±1.69**	**1.63±0.58**	**1.96±0.87**	**5.49±1.15**	**6.65±2.44**	2.53±0.50	**1.13±0.22**	**1.36±0.51**	4.92±0.87
September*2013	**3.09±0.67**	**2.97±0.61**	**1.14±0.24**	**1.11±0.34**	**4.22±0.69**	**4.08±0.93**	2.75±0.59	**0.89±0.14**	**0.85±0.14**	4.75±0.48
A*S*Y										
Young*June*2011	3.04±0.33	**2.81±0.59**	1.15±0.13	1.06±0.23	4.18±0.45	**3.87±0.82**	2.65±0.10	1.08±0.16	**0.99±0.18**	3.91±0.31
Young*June*2013	3.62±0.75	**3.39±0.89**	1.65±0.92	1.56±1.00	5.27±1.63	**4.96±1.86**	2.46±0.59	1.00±0.12	**0.93±0.09**	5.29±1.65
Young*September*2011	4.23±0.47	**5.68±1.75**	1.81±0.52	2.40±0.86	6.03±0.88	**8.08±2.44**	2.46±0.51	1.26±0.23	**1.69±0.53**	4.84±0.76
Young*September*2013	3.26±0.29	**3.05±0.44**	1.25±0.22	1.19±0.37	4.50±0.42	**4.24±0.78**	2.66±0.36	0.94±0.15	**0.88±0.11**	4.82±0.53
Old*June*2011	2.55±0.30	**3.07±0.84**	0.88±0.14	1.06±0.34	3.43±0.44	**4.13±1.15**	2.93±0.19	0.86±0.09	**1.01±0.18**	4.03±0.47
Old*June*2013	2.59±0.54	**2.73±0.39**	1.32±0.40	1.41±0.51	3.91±0.66	**4.15±0.63**	2.11±0.59	0.81±0.08	**0.86±0.12**	4.89±0.97
Old*September*2011	3.51±0.59	**3.70±0.89**	1.45±0.61	1.52±0.67	4.96±1.17	**5.22±1.49**	2.60±0.52	0.99±0.12	**1.04±0.21**	5.01±1.02
Old*September*2013	2.92±0.59	**2.89±0.78**	1.03±0.23	1.03±0.32	3.95±0.81	**3.92±1.08**	2.84±0.21	0.84±0.12	**0.83±0.18**	4.69±0.47
F values and probability levels										
Age (A)	**25.324*****	**7.799****	**6.091***	3.928n.s.	**17.183*****	**7.023***	0.348n.s.	**31.335*****	**9.404****	0.074n.s.
Season (S)	**17.154*****	**13.485****	1.326n.s.	2.989n.s.	**8.696****	**9.679****	0.316n.s.	**4.437***	**7.352****	1.961n.s.
Year (Y)	3.443n.s.	**12.377****	0.007n.s.	2.021n.s.	1.197n.s.	**8.298****	0.180n.s.	**17.910*****	**26.766*****	**4.833***
A*S	0.807n.s.	3.678n.s.	0.004n.s.	2.235n.s.	0.294n.s.	3.523n.s.	0.360n.s.	0.074n.s.	**7.370****	0.130n.s.
A*Y	0.115n.s.	0.995n.s.	0.028n.s.	0.886n.s.	0.011n.s.	1.094n.s.	0.174n.s.	1.884n.s.	**4.416***	0.898n.s.
S*Y	**18.480*****	**16.486*****	**16.337*****	**18.329*****	**20.778*****	**19.796*****	0.001n.s.	**5.858***	**11.554****	8.854n.s.
A*S*Y	3.271n.s.	**9.138****	0.178n.s.	2.112n.s.	1.550n.s.	**6.625***	0.119n.s.	0.905n.s.	**8.230****	0.063n.s.

Tab. 3: Values (mean ± standard deviation) for phenolic, TBARS, protein and thiols content, and electrolyte leakage, of *Salvia officinalis* leaves and probability levels of the effects of Age, Season and Year, as determined by ANOVA. ns: not significant. In bold, results that showed to be affected by the studied factors and/or their interaction.

	Total phenolics (mg GA/dm^2)	Total phenolics (mg GA/g)	TBARS (nmol/cm^2)	TBARS (nmol/g)	Protein (mg/dm^2)	Protein (mg/g)	Thiols (nmol/mg protein)	Electrolytes leakage (%)
Age (A)								
Young	39.44±12.67	37.49±12.55	**3.02±1.78**	**313.89±191.84**	136.45±151.08	146.26±176.92	52.59±60.46	14.87±2.48
Old	36.31±11.20	40.12±10.34	**1.43±0.49**	**153.19±52.29**	128.72±93.58	136.33±97.18	41.69±34.76	15.12±4.90
Season (S)								
June	38.49±9.86	38.99±10.48	2.18±1.78	215.25±161.92	145.72±157.12	150.33±163.24	**76.36±54.92**	14.70±2.31
September	37.25±13.89	38.62±12.57	2.27±1.23	251.83±161.05	119.44±81.07	132.26±118.23	**17.93±12.13**	15.18±4.53
Year (Y)								
2011	**32.67±12.38**	**35.29±11.52**	2.96±1.75	**312.12±179.55**	118.98±103.61	136.88±133.29	52.53±62.70	**12.88±2.72**
2013	**43.08±9.05**	**42.31±10.48**	1.49±0.73	**145.96±69.23**	146.19±143.17	145.71±151.62	41.76±30.55	**15.63±3.94**
A*S								
Young*June	39.66±9.91	36.32±9.11	2.99±2.19	276.02±205.43	152.13±196.41	149.44±207.69	88.12±68.75	**15.42±2.54**
Young*September	39.21±15.28	38.66±15.48	3.04±1.31	351.76±175.50	120.77±89.85	143.07±146.72	17.07±11.61	**14.53±2.47**
Old*June	37.34±9.98	41.66±11.35	1.36±0.58	154.48±64.40	139.32±111.15	151.21±109.23	64.60±34.79	**13.99±2.31**
Old*September	35.29±12.55	38.57±9.32	1.51±0.39	151.90±38.72	118.11±74.19	121.45±84.35	18.79±12.95	**15.83±5.95**
A*Y								
Young*2011	36.31±14.57	35.13±13.96	4.34±1.40	**469.21±131.12**	111.28±99.56	134.63±153.48	58.93±77.53	**14.97±2.03**
Young*2013	42.56±9.93	39.85±10.89	1.69±0.90	**158.67±86.05**	161.63±189.50	157.88±202.09	46.26±38.19	**14.84±2.65**
Old*2011	29.04±8.75	35.45±8.90	1.57±0.50	**173.04±51.46**	126.67±110.23	139.13±114.68	46.13±45.03	**10.80±1.29**
Old*2013	43.59±8.36	44.78±9.76	1.29±0.45	**133.35±46.49**	130.75±77.08	133.54±79.69	37.26±20.68	**16.42±4.85**
S*Y								
June*2011	31.71±8.86	**32.52±8.25**	3.27±1.93	319.55±166.08	108.56±98.76	114.43±98.16	**96.05±63.51**	n.a.
June*2013	45.29±4.89	**45.46±8.35**	1.09±0.55	110.95±58.59	182.90±195.75	186.23±206.61	**56.68±37.04**	14.70±2.31
September*2011	33.64±15.38	**38.07±13.77**	2.65±1.54	322.69±197.57	129.40±110.46	159.33±161.27	**9.01±7.21**	12.88±2.72
September*2013	40.87±11.61	**39.16±11.67**	1.90±0.67	180.97±62.10	109.48±34.19	105.19±36.32	**26.85±9.08**	16.56±4.89
A*S*Y								
Young*June*2011	33.93±10.99	30.21±7.26	4.84±1.44	443.94±148.06	98.90±69.49	94.73±68.47	109.71±83.25	n.a.
Young*June*2013	45.38±3.84	42.43±6.32	1.14±0.64	108.09±63.68	205.37±267.13	204.16±284.44	66.53±46.19	15.42±2.55
Young*September*2011	38.68±17.92	40.06±17.60	3.84±1.25	494.47±116.03	123.66±126.72	174.53±205.31	8.14±7.37	14.97±2.04
Young*September*2013	39.75±13.36	37.27±14.12	2.24±0.80	209.04±77.38	117.89±34.97	111.61±41.38	25.99±7.23	14.26±2.76
Old*June*2011	29.48±6.02	34.83±9.01	1.69±0.49	195.16±42.70	118.21±125.93	134.13±114.24	82.38±35.88	n.a.
Old*June*2013	45.19±6.04	48.49±9.39	1.03±0.47	113.81±57.29	160.43±97.96	168.29±99.13	46.82±24.19	13.99±2.31
Old*September*2011	28.59±11.28	36.08±9.36	1.45±0.52	150.91±52.28	135.14±100.06	144.12±114.24	9.89±7.44	10.80±1.29
Old*September*2013	41.99±10.37	41.06±9.17	1.56±0.23	152.89±21.84	101.08±33.47	98.78±31.94	27.70±11.07	18.85±5.58
F values and probability levels								
Age (A)	1.322n.s.	0.934n.s.	**58.465*****	**61.502*****	0.075n.s.	0.059n.s.	1.354n.s.	1.784n.s.
Season (S)	0.210n.s.	0.019n.s.	0.224	3.187n.s.	0.248n.s.	0.680n.s.	**38.946*****	3.264n.s.
Year (Y)	**14.694*****	**6.687***	**49.975*****	**73.076*****	0.059n.s.	0.729n.s.	1.323n.s.	**9.590****
A*S	0.087n.s.	1.004n.s.	0.052	0.365n.s.	0.104n.s.	0.025n.s.	1.818n.s.	**8.603****
A*Y	2.333n.s.	0.720n.s.	**32.785*****	**43.716*****	0.158n.s.	0.527n.s.	0.041n.s.	**13.665****
S*Y	1.367n.s.	**4.761***	**11.963*****	2.663n.s.	3.013n.s.	2.186n.s.	**9.334****	n.a.
A*S*Y	0.551n.s.	0.340n.s.	2.530	0.161n.s.	0.409n.s.	0.080n.s.	0.042n.s.	n.a.

Season and Year factors showed influence in the results reported by leaf weight (Tab. 3). Considerable variation of the content on phenolics present in aromatic and medicinal plants has been reported by other authors (Kofidis et al., 2007). The relationship to harvest season, with increase in summer months, has been linked to their protective properties against excessive UV-B radiation that might reach mesophyll chloroplasts and nuclei, therefore shielding leaf cells from structural and functional damages (Manukyan, 2013). However, our results do not show this behaviour, and the detected influence caused by the Season*Year interaction appears to be more likely caused by the year factor. As referred before, total phenolic compounds present in the leaves of sage were influenced by the year of study, with higher values recorded in 2013, with the same pattern observed for electrolyte leakage. These results appear to indicate that in 2013, the plants were under increased stress conditions, as it is well established that phenolics act as defense mechanisms against stress conditions (e.g. Manukyan, 2013), while electrolyte leakage is used to estimate membrane integrity (Rolny et al., 2011). Climatic data concerning precipitation and temperature show that 2011 was one of the hottest years in Portugal since 1930, and that precipitation was also considerably lower than common records (IPMA, 2015), while 2013 was considered an average year. This information about climatic data gives more consistency with the results observed for TBARS. This methodology allows an overview regarding peroxidation of membrane, and usually, higher values indicate a higher exposure to stress, which apparently, and as stated above, occurred in 2011. Therefore, some other factors would have been implicated in this higher amount of phenolics and of electrolyte leakage. By one hand, the higher values of total phenolics in 2013, quantified using the Folin-Ciocalteu method can be due to the higher amount of sugars found is this year, compounds, that among others, are known to interfere with this methodology (Prior et al., 2005). Sugars may also be associated to the values of electrolyte leakage, as they have been correlated to an increase in this parameter (Shi et al., 2012), although they are known as key osmolytes, improving stress tolerance by protecting and stabilizing membranes.

The age factor also significantly influenced the TBARS content (expressed as nmol/g), with higher values recorded in young leaves. Although several reports indicate that the amount of these compounds may be higher in adult leaves of several species (Lepeduš et al., 2011), higher lipid peroxidation measured by the TBARS assay in young leaves has been reported (Carvalho et al., 2015). Interestingly, the total carotenoid content in young leaves was also higher (Tab. 2), and it can be argued that their accumulation in young leaves can be related not to their photosynthetic role, but instead to their known protective antioxidant characteristics (Krinsky, 1989).

There is a lack of information about how the presence of phenolics can influence the processing of aromatic plants. It can be expected that leaves with higher content of phenolics can be less susceptible to oxidation, as those compounds are proved to be antioxidants (e.g. Gião et al., 2013). The presence of these compounds can also influence leaf sensorial attributes, as they have associated two main descriptors, namely bitterness and astringency, which are linked to negative consumer reactions (Lesschaeve and Noble, 2005). For thiol content (Tab. 3), a strong influence of season was found, with samples collected in June presenting higher values than those recorded in September. These compounds act like intracellular antioxidants, either through scavenging free radicals or by enzymatic reactions, and some of them, although being water-soluble, are able to protect biological membranes against lipid peroxidation (Mascio et al., 1991). Interestingly, none of the other studied parameters that evaluate oxidation processes show this behavior pattern of higher values in June. In this month, although temperatures were similar to those recorded in September (average of 18.2 °C in June and 19.6 °C in September), rain was considerably lower (average of 5.6 mm in June and 67.8 mm in September), and, furthermore, total radiation was significantly higher (average of 27.6 kj/m^2 in June and 16.9 kj/m^2 in September). Hence, this increase in thiols concentration can be a response to those stresses, in order to reduce oxidation (Mascio et al., 1991) and cell damage.

Conclusions

This work allowed a detailed characterization of several biochemical parameters of *Salvia officinalis* L. var. *purpurascens,* as well as an evaluation of their dynamics, considering developmental stage, season and year factors. This latter factor was the one that exerted a significant influence in a higher number of parameters, situation that is likely linked to differences in climatic conditions (considerable difference of precipitation between the years of study, but also of temperature values), while, on the other hand, the developmental stage of leaves influenced almost only the content on photosynthetic pigments. These results are key to understand biochemical variations occurring in Salvia *officinalis* L. var. *purpurascens*, and helpful when designing cultivation strategies for this specific plant.

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