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Leaf morpho-physiological dynamics in *Salvia officinalis* L. var. *purpurascens*

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Abstract: *Salvia officinalis* L. var. *purpurascens* is one of the most insufficiently studied varieties of the genus *Salvia*. Hence, the study and characterization of this plant, as well as the monitoring of changes occurring during leaf ontogeny, are of great importance and interest. For this purpose, young and adult leaves of *Salvia officinalis* L. var. *purpurascens* collected in two different seasons and in two different years were analyzed, considering morpho-anatomical traits but also leaf gas exchange and chlorophyll a fluorescence parameters. Leaf age was found to have significant effects on several of the analyzed parameters, including the parenchyma, mesophyll, xylem, and phloem thickness, as well as transpiration rate (E) and maximum photochemical efficiency of PSII (F_v/F_m). On the other hand, considerable differences were found for several characteristics of *Salvia officinalis* caused by season, e.g., leaf area, vessel dimensions, stomatal conductance (g_s), waxes, E, and intrinsic water-use efficiency (A/g_s). The year of harvest also resulted in significant variations in several parameters, such as leaf area and leaf mass per area (LMA), and palisade and spongy parenchyma thickness. These results show the dynamics of *Salvia officinalis* leaf traits, presently poorly known, and are further helpful when aiming towards optimization of characteristics of cultivated sage.

Key words: *Salvia officinalis* L. var. *purpurascens*, morphological traits, leaf gas exchange, chlorophyll a fluorescence

1. Introduction

Herbs and spices have been used by humans since ancient times due to their culinary and medicinal properties, and, more recently, the interest in this class of plants has increased due to their recognized and studied beneficial health effects (Gan et al., 2010). Indeed, the consumption of herbs and spices has been linked to the prevention of several diseases (Albano and Miguel, 2011), associated with their content of bioactive compounds (Scalbert et al., 2005). Common sage or garden sage (*Salvia officinalis* L., family Lamiaceae) is a small evergreen perennial with origin in the Mediterranean region and Asia Minor, being an important medicinal and aromatic plant used in folk medicine for centuries (Seidler-Łożykowska et al., 2015). The leaves of *Salvia officinalis* are rich in essential oils, they contain several phytochemicals (Seidler-Łożykowska et al., 2015), and they are known for having several medicinal uses, but also as herbal tea and spices and in cosmetics and perfumery (Alizadeh and Shaabani, 2012). Worldwide, the global trade of herb-based products was worth an estimated $60,000 million in 2000 (World Health Organization, 2003), with increasing demand to search for new bioactive compounds from these plants. This, in consequence, results in higher concern, regarding both safety and quality, but also regarding the collection of wild material to be traded commercially. This can result in two major problems: i) safety issues, as plants may be contaminated by other species or plant parts through misidentification, accidental contamination, or intentional adulteration; ii) overharvesting, with destructive harvesting techniques (World Health Organization, 2003). Therefore, aromatic and medicinal plants are now largely being inserted in cultivation systems to, on one hand, help the sustainability of those plant species, and, on the other hand, to monitor and optimize conditions for higher plant quality (Schippmann et al., 2006). Therefore, it is of great importance to study different factors that may influence characteristics of aromatic and medicinal plants, in order to produce high quality products, from both the producers' as well from the consumers' point of view. Some available works regarding *Salvia officinalis* L. focused on variations in growth parameters, volatile composition, essential oils, and phenolics caused by the type of cultivation (field or greenhouse) (Yi and Wetzstein, 2010), saline stress (Taârit

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et al., 2012), low light conditions (Mapes and Xu, 2014), or water deficit (Bettaieb et al., 2011), but *Salvia officinalis* L. var. *purpurascens*, one of the many varieties of *Salvia officinalis*, is currently insufficiently studied. Hence, this work aimed to identify morphological and physiological variations caused by leaf age, season, or year in *Salvia officinalis* L. var. *purpurascens*.

2. Materials and methods

2.1. Plant material

Salvia officinalis L. var. *purpurascens* plants were grown in the Botanical Garden of UTAD, Vila Real (41°19′N, 7°44′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 (nonhumic litholic) derived from shale. It 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. Twelve plants were selected and healthy leaves of each age (young, presenting the characteristic purple coloration (Karabacak et al., 2009) and adult), sampling date (June and September), and year (2011 and 2013) were sampled from 4-year-old plants, and eight repetitions of all methodologies were performed in randomly selected leaves. The study was conducted between 2011 and 2013, but only the results from those two years (2011 and 2013) were considered, as they presented considerable differences regarding several climatic conditions (namely precipitation and temperature, Figure), while conditions were similar between 2012 and 2013.

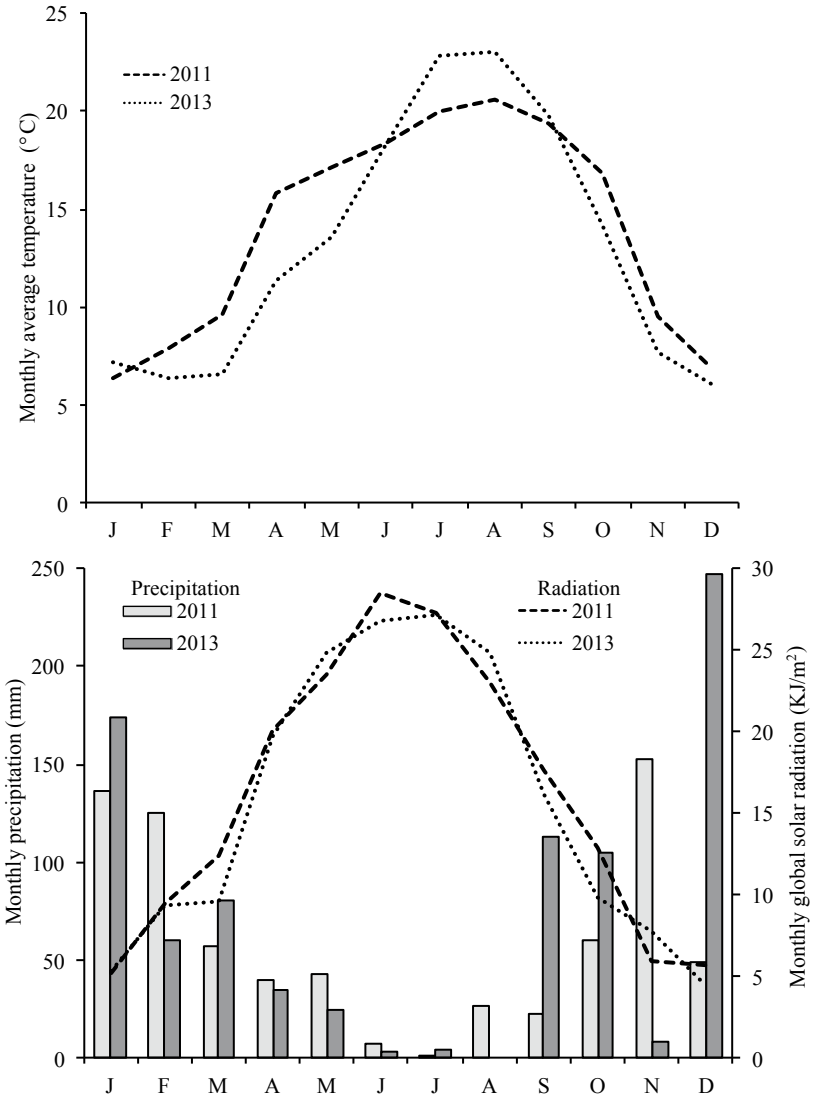

Figure. Average monthly temperature (°C), total monthly precipitation (mm), and global solar radiation (kJ/m^2) for 2011 and 2013.

2.2. Leaf morpho-physiological determinations

In each year on each sampling date, in young and adult leaves, ten samples were recovered, and the following parameters were evaluated: leaf area (WinDIAS Leaf Area Meter System software, Delta–T devices Ltd., Cambridge, UK), fresh mass (g), fresh mass at full turgor (after immersion of leaves in demineralized water for 24 h in the dark, at 4 °C), and dry mass (after drying in oven at 70 °C to a constant weight). Leaf mass per area and two leaf moisture indices (relative water content (RWC) and succulence) were evaluated according to Gonçalves et al. (2009). For the quantification of soluble cuticular waxes (in 2013 only), leaf area was measured and samples were stirred for 2 min in a mixture of chloroform and methanol 3:1 (50 mL). The solution was filtered and allowed to evaporate to leave only the dry material.

2.3. Leaf tissue thickness determinations

From the same plants, and in each sampling date and year, two tissue samples from each leaf (n = 8) were taken midway between the leaf edge and the midvein to measure leaf blade, upper and lower epidermis, including cuticles, and palisade and spongy parenchyma, under a light microscope (Olympus IX 51, Olympus Optical Co., GmbH, Hamburg, Germany), using the program Cell* (Soft Imaging System GmbH, Hamburg, Germany). In the leaves of 2013, and using the same tissue samples, leaf vessel dimensions, namely vessel area, perimeter, and vascular bundle width, as well as phloem and xylem thickness were measured.

2.4. Leaf gas exchange and chlorophyll a fluorescence

Leaf gas exchange was measured in 8 leaves, on two sampling dates, in 2011, using an Infrared Gas Analyzer System LCPro-SD (ADC Bioscientific Ltd., UK) and the equations of von Caemmerer and Farquhar (1981) were used for estimation of photosynthetic rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E). Intrinsic water-use efficiency was calculated as the ratio of A to g_s (A/g_s). Measurements were performed under an average photosynthetic photon flux (PPFD) of 1479 μmol photons m^{-2} s^{-1} and external CO_2 concentration of 379.9 ppm. A portable chlorophyll pulse amplitude modulated fluorometer (FMS2, Hansatech Instruments, Norfolk, UK) was used to determine maximum photochemical efficiency of PSII in 8 dark-adapted leaves (F_v/F_m), minimal (F_0) and maximal fluorescence (F_m) at open and closed PSII reaction centers, respectively, and variable fluorescence (F_v). Measurements were performed in the same leaves as for leaf gas exchange, but, before measurements, the leaves were dark-adapted for 30 min in a clamp cuvette, and a low intensity pulsed measuring light source was used for F_0 and a pulse saturating light (0.7 s pulse of 15,000 μmol photons m^{-2} s^{-1} of white light) when all reactions centers were closed for F_m. After F_v/F_m estimation, a 20-s exposure to actinic light (1500 μmol m^{-2} s^{-1}), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (15,000 μmol m^{-2} s^{-1}) for 0.7 s to establish F'_m. The sample was then shaded for 5 s with a far-red light source to determine F'_0. Using data of those measurements, several fluorescence attributes were calculated according to Bilger and Schreiber (1986) and Genty et al. (1989), namely photochemical quenching (nonphotochemical quenching (and efficiency of electron transport as a measure of the quantum effective efficiency of PSII (). The apparent electron transport rate (ETR) was estimated as , where PPFD is the photosynthetic photon flux density incident on the leaf and 0.5 is the factor assuming equal distribution of energy between the two photosystems, using leaf absorbance of 0.84 because it is the most common value for C3 plants (Björkman and Demmig, 1987). All measurements were performed in midday sun.

2.5. Statistical analysis

Data are presented as mean ± standard deviation, and differences among means were determined by analysis of variance (ANOVA), using SPSS, version 19.0 (IBM Corporation, New York, NY, USA). 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 test with Lilliefors correction (if n >50) or the Shapiro–Wilk's test (if n < 50), and the Levene test, respectively. Regression analyses were performed for all the considered leaf traits, but only those with $R^2 > 0.5$ are presented.

3. Results and discussion

3.1. Leaf morpho-anatomical determinations

Morphological and anatomical parameters of *Salvia officinalis* leaves were influenced by the tested factors (age, season, and year) in different ways (Table 1). Leaf age resulted in significant variation in the area (higher in adult leaves) and waxes content of leaves (higher in young leaves). Considering both the season and year factors, harvest resulted in significant changes in the leaf area (higher in June and 2011, compared to September and 2013) and succulence (higher in September and 2011), while, for the season factor, changes were also found in the wax content (higher in September than in June) and, for the year factor, in LMA (higher values in 2011, compared to 2013). Both seasonal and annual variations in leaf are most likely associated with differences in climatic conditions (Poorter et al., 2009). For instance, leaf thickness has been negatively (Chabot and Chabot, 1977; Vile et al., 2012) or positively (Meier and Leuschner, 2008) correlated with increased temperature, while specific leaf area has been negatively correlated with increased temperature (Chabot

Table 1. Values (mean ± standard deviation) for morpho-anatomical traits of *Salvia officinalis* leaves and probability levels of the effects of age, season, and year as determined by ANOVA. ns: not significant; n.a. data not available. In bold, results shown to be affected by the studied factors and/or their interaction.

	Leaf area (cm^2)	LMA ($g\ m^{-2}$)	RWC (%)	Waxes ($\mu g\ cm^{-2}$)	Succulence ($mg\ cm^{-2}$)
Age (A)					
Young	**9.56 ± 2.20**	86.86 ± 25.48	91.15 ± 4.79	**754.56 ± 109.07**	0.23 ± 0.06
Adult	**13.68 ± 6.69**	84.23 ± 18.09	89.01 ± 8.24	**609.51 ± 140.20**	0.24 ± 0.06
Season (S)					
June	**15.84 ± 4.84**	88.80 ± 19.81	88.49 ± 4.22	**637.69 ± 154.57**	**0.22 ± 0.04**
September	**7.40 ± 3.32**	82.29 ± 23.78	91.67 ± 8.38	**726.38 ± 120.82**	**0.25 ± 0.07**
Year (Y)					
2011	**14.29 ± 6.48**	**104.22 ± 22.77**	89.91 ± 2.92	n.a.	**0.27 ± 0.06**
2013	**10.02 ± 4.98**	**74.34 ± 11.39**	90.19 ± 8.31	682.04 ± 144.11	**0.22 ± 0.06**
A × S					
Young × June	**13.07 ± 2.51**	**94.39 ± 21.16**	89.98 ± 2.22	715.05 ± 112.14	0.22 ± 0.04
Young × September	**6.05 ± 1.97**	**79.34 ± 27.80**	92.33 ± 6.28	794.08 ± 95.22	0.24 ± 0.07
Adult × June	**18.60 ± 5.08**	**83.22 ± 17.23**	87.01 ± 5.21	560.34 ± 156.72	0.23 ± 0.03
Adult × September	**8.75 ± 3.87**	**85.24 ± 19.43**	91.01 ± 10.23	658.68 ± 107.54	0.26 ± 0.08
A × Y					
Young × 2011	**9.95 ± 2.20**	104.94 ± 31.61	89.06 ± 2.92	n.a.	0.25 ± 0.08
Young × 2013	**9.32 ± 5.08**	76.02 ± 12.26	92.41 ± 5.29	754.56 ± 109.07	0.21 ± 0.03
Adult × 2011	**18.63 ± 6.46**	103.49 ± 9.15	90.76 ± 2.77	n.a.	0.28 ± 0.03
Adult × 2013	**10.71 ± 4.91**	72.66 ± 10.51	87.96 ± 10.16	609.51 ± 140.20	0.22 ± 0.07
S × Y					
June × 2011	17.89 ± 7.15	108.95 ± 12.02	**90.54 ± 2.19**	n.a.	0.25 ± 0.02
June × 2013	14.60 ± 2.09	76.71 ± 12.11	**87.26 ± 4.69**	637.69 ± 154.57	0.20 ± 0.03
September × 2011	10.68 ± 2.86	99.48 ± 29.85	**89.28 ± 3.48**	n.a.	0.28 ± 0.08
September × 2013	5.44 ± 1.53	71.97 ± 10.40	**93.11 ± 10.09**	726.38 ± 120.82	0.24 ± 0.07
A × S × Y					
Young × June × 2011	**11.60 ± 1.86**	116.59 ± 11.42	89.98 ± 1.98	n.a.	0.26 ± 0.02
Young × June × 2013	**13.94 ± 2.51**	81.07 ± 12.16	89.98 ± 2.45	715.05 ± 112.14	0.19 ± 0.03
Young × September × 2011	**8.30 ± 0.82**	93.28 ± 41.74	88.14 ± 3.58	n.a.	0.25 ± 0.11
Young × September × 2013	**4.70 ± 0.83**	70.97 ± 10.62	94.84 ± 6.32	794.08 ± 95.22	0.24 ± 0.02
Adult × June × 2011	**24.18 ± 3.76**	101.31 ± 6.87	91.01 ± 2.43	n.a.	0.25 ± 0.02
Adult × June × 2013	**15.26 ± 1.39**	72.36 ± 10.94	84.55 ± 4.91	560.34 ± 156.72	0.21 ± 0.03
Adult × September × 2011	**13.07 ± 1.91**	105.69 ± 11.21	90.41 ± 3.27	n.a.	0.30 ± 0.02
Adult × September × 2013	**6.17 ± 1.75**	72.96 ± 10.65	91.37 ± 12.97	658.68 ± 107.54	0.23 ± 0.09
Probability levels					
Age (A)	**0.000**	n.s.	n.s.	**0.001**	n.s.
Season (S)	**0.000**	n.s.	n.s.	**0.025**	**0.045**
Year (Y)	**0.000**	**0.000**	n.s.	n.a.	**0.001**
A × S	**0.000**	**0.025**	n.s.	n.s.	n.s.
A × Y	**0.000**	n.s.	n.s.	n.a.	n.s.
S × Y	n.s.	n.s.	**0.037**	n.a.	n.s.
A × S × Y	**0.000**	n.s.	n.s.	n.a.	n.s.

and Chabot, 1977; Vile et al., 2012), the same as LMA, palisade mesophyll tissue, and the number of cell layers comprising the leaf mesophyll palisade (Cohu et al., 2014). In Portugal, in 2011, higher average air temperatures were recorded, compared to 2013 (IPMA, 2015), which can help to explain the annual variation recorded for leaf morpho-anatomical parameters. The significant variation in leaf area and succulence values detected between different seasons and years of sampling can also be related to precipitation. In fact, the rainfall amount in 2011 was considerably lower than in 2013, which can lead to an increase in succulence in the leaves, as they use water storage and leaf area reduction to avoid desiccation (Larcher, 1995). This is also true for values recorded in September, as higher succulence and lower leaf area indicate that plants are adjusting to decreased water availability. Similarly, higher values of LMA have been linked to drought conditions, as a consequence of an increase in density or thickness of foliar tissue, which usually occurs when the costs of the assimilatory apparatus increase (Centritto, 2002). In fact, our results show variations in values of LMA that are probably linked to higher tissue thickness, also detected, further confirmed by significant correlations found in some of those parameters, namely palisade parenchyma (0.462, P = 0.000), spongy parenchyma (0.295, P = 0.018), mesophyll (0.401, P = 0.001), lower epidermis+cuticle (0.529, P = 0.000), and leaf blade (0.385, P = 0.002).

Regarding waxes, no data were found regarding their presence in sage, although variations caused by leaf age and date of sampling have been found in other plants (Kahmen et al., 2011). The detection of higher wax content in leaves collected in September may be a mechanism to reduce water loss by transpiration through the leaf blade surface, caused by high temperatures recorded in the months previous to the sampling date (IPMA, 2015). In contrast, the changes between young and adult leaves can be due to the rapid expansion of leaf area (although not significant different, but with higher values for adult leaves) that wax synthesis was unable to accompany (Bringe et al., 2006). The interaction of two of the studied factors also resulted in significant variation in leaf morpho-anatomical features. The interaction of the age and season factors significantly affected leaf area and LMA, age and year affected leaf area, and season and year interaction only significantly influenced RWC. Nevertheless, it should be noted that, due to the lack of data for waxes in 2011, the interaction of factors cannot be evaluated. The three-way interaction of age × season × year also had significant effects on leaf area.

3.2. Leaf tissue thickness determinations

The year of sampling was the factor that caused more significant variations in leaf tissue dimensions (Table 2), with annual variations detected in almost all parameters evaluated, with the exception of the thickness of the upper epidermis. For most of them, values were higher in 2011 (palisade, spongy parenchyma, mesophyll, lower epidermis, and leaf blade thickness), while the palisade/spongy parenchyma ratio was the only parameter of those significantly influenced by the year factor that presented higher values in 2013. Leaf age also influenced tissue thickness, with higher values recorded for adult leaves compared to young ones, namely for thickness of spongy and palisade parenchyma, mesophyll, and lower epidermis with cuticle, which subsequently influenced leaf blade. Data regarding these specific characters in leaves of *Salvia officinalis* are very scarce. Nasta et al. (2014) showed values of leaf thickness ranging from 0.38 to 0.54 mm, for fully expanded leaves, while Yi and Wetzstein (2010) reported similar values (471 ± 51 μm or 517 ± 47 μm), depending on whether plants were grown in field or greenhouse conditions. The data recorded in our work show considerably less thick leaves than those, most likely due to cultivation conditions. In the work by Nasta et al. (2014), although no rainfall occurred, plants were drip-irrigated, and in Yi and Wetzstein (2010) no information is found about irrigation or rainfall. The work by Nasta et al. (2014) also shows differences in leaf thickness according to their age. Hence, their results showed an increase in this parameter, with thickness of leaf increasing from 0.41 ± 0.02 mm to 0.46 ± 0.02 mm in leaves 57 days older. This same pattern of higher leaf thickness but also thickness of spongy and palisade parenchyma with increasing leaf age has been detected in other species, like *Prunus persicae* or *Cistus incanus* (Gratani and Bombelli, 2000), although this does not occur for all plant species, and it is true only until leaf expansion occurs, stopping and decreasing progressively as leaf age increases. Seasonal variations were also found, with the palisade/spongy parenchyma ratio significantly higher in leaves collected in June. In contrast, spongy parenchyma and mesophyll thickness and succulence were higher in September. Although no data were found regarding seasonal variations in leaf traits in *Salvia officinalis*, it is known that leaves can suffer variations in these characteristics in response to several factors (Witkowski and Lamont, 1991), namely climatic conditions, which can result in a response from the leaves to counteract harmful effects of high irradiance or temperature, or low water availability (Letts et al., 2012). Indeed, seasonal variations in morphologic leaf parameters have been reported in some other plants, like *Mentha spicata* or *Clinopodium vulgare* (Kofidis et al., 2007, 2011). Variations in leaf tissue thickness may have a role in the light capture profile of leaves, leading to possible changes in photosynthesis. Higher values of palisade parenchyma thickness will be related to a higher ability to enable light penetration to chloroplasts, resulting in higher photosynthetic activity, to which adult leaves

Table 2. Values (mean ± standard deviation) for leaf tissue dimensions of *Salvia officinalis* and probability levels of the effects of age, season, and year as determined by ANOVA. ns: not significant. In bold, results shown to be affected by the studied factors and/or their interaction.

	Upper epidermis + cuticle (µm)	Palisade parenchyma (µm)	Spongy parenchyma (µm)	Mesophyll (µm)	Lower epidermis + cuticle (µm)	Leaf blade (µm)	PP/SP
Age (A)							
Young	18.61 ± 2.88	**66.89 ± 12.00**	**46.79 ± 15.37**	**115.03 ± 23.29**	**11.09 ± 4.95**	**149.08 ± 25.32**	1.56 ± 0.53
Adult	18.14 ± 3.09	**79.09 ± 13.75**	**59.39 ± 16.16**	**140.19 ± 25.46**	**13.21 ± 4.03**	**177.19 ± 26.65**	1.40 ± 0.37
Season (S)							
June	18.22 ± 3.05	74.02 ± 12.77	**49.72 ± 14.64**	**123.84 ± 24.00**	12.24 ± 4.82	160.49 ± 26.26	**1.59 ± 0.47**
September	18.53 ± 2.94	71.58 ± 15.42	**55.72 ± 18.42**	**130.13 ± 29.97**	11.99 ± 4.49	164.49 ± 32.19	**1.39 ± 0.44**
Year (Y)							
2011	18.79 ± 2.11	**85.12 ± 11.87**	**67.33 ± 13.79**	**152.45 ± 22.50**	**17.55 ± 2.04**	**188.80 ± 22.44**	**1.31 ± 0.34**
2013	18.19 ± 3.31	**66.92 ± 11.19**	**46.02 ± 13.69**	**115.18 ± 20.49**	**9.55 ± 3.03**	**150.23 ± 23.79**	**1.57 ± 0.49**
A × S							
Young × June	**18.99 ± 2.93**	**69.82 ± 11.72**	44.93 ± 14.35	**114.75 ± 22.44**	11.24 ± 4.97	**150.81 ± 25.30**	1.68 ± 0.52
Young × September	**18.23 ± 2.81**	**63.96 ± 11.70**	48.66 ± 16.31	**115.30 ± 24.41**	10.94 ± 4.99	**147.34 ± 25.55**	1.44 ± 0.52
Adult × June	**17.32 ± 2.98**	**78.98 ± 12.32**	55.37 ± 13.04	**134.58 ± 21.46**	13.41 ± 4.43	**171.93 ± 22.82**	1.49 ± 0.39
Adult × September	**18.83 ± 3.06**	**79.19 ± 15.02**	62.79 ± 17.86	**144.95 ± 27.80**	13.06 ± 3.71	**181.65 ± 29.05**	1.33 ± 0.34
A × Y							
Young × 2011	19.09 ± 2.61	**76.08 ± 7.25**	62.28 ± 12.49	138.36 ± 16.17	**17.51 ± 1.94**	174.96 ± 17.42	1.29 ± 0.43
Young × 2013	18.39 ± 2.99	**62.81 ± 11.46**	39.91 ± 15.37	104.66 ± 17.86	**8.24 ± 2.66**	137.58 ± 19.02	1.68 ± 0.53
Adult × 2011	18.50 ± 1.45	**94.17 ± 8.04**	72.38 ± 13.40	166.55 ± 18.93	**17.59 ± 2.18**	202.65 ± 18.05	1.33 ± 0.34
Adult × 2013	17.95 ± 3.66	**71.56 ± 8.95**	52.89 ± 13.32	127.02 ± 16.49	**11.03 ± 2.75**	164.47 ± 20.42	1.43 ± 0.42
S × Y							
June × 2011	**19.56 ± 2.59**	84.86 ± 9.21	**61.35 ± 10.06**	146.21 ± 16.42	17.78 ± 2.71	183.55 ± 16.90	1.42 ± 0.21
June × 2013	**17.56 ± 3.07**	68.59 ± 10.71	**43.89 ± 13.06**	112.66 ± 18.84	9.46 ± 2.83	148.96 ± 22.25	1.69 ± 0.55
September × 2011	**18.04 ± 1.08**	85.39 ± 14.24	**73.31 ± 14.59**	158.69 ± 26.14	17.32 ± 1.04	194.05 ± 26.19	1.22 ± 0.42
September × 2013	**18.75 ± 3.44**	65.44 ± 11.51	**47.91 ± 14.07**	117.43 ± 21.78	9.63 ± 4.49	151.36 ± 25.24	1.46 ± 0.43
A × S × Y							
Young × June × 2011	20.15 ± 3.28	79.21 ± 5.54	**60.23 ± 10.09**	**139.44 ± 14.28**	17.49 ± 2.64	177.08 ± 16.02	1.34 ± 0.18
Young × June × 2013	18.47 ± 2.68	65.65 ± 11.36	**38.13 ± 10.09**	**103.78 ± 18.61**	8.46 ± 2.66	139.13 ± 19.16	1.84 ± 0.55
Young × September × 2011	18.03 ± 1.05	72.95 ± 7.61	**64.32 ± 14.68**	**137.28 ± 18.45**	17.52 ± 0.93	172.83 ± 19.17	1.25 ± 0.59
Young × September × 2013	18.32 ± 3.32	59.97 ± 11.04	**41.69 ± 11.56**	**105.54 ± 20.12**	8.02 ± 2.69	136.01 ± 19.12	1.53 ± 0.47
Adult × June × 2011	18.97 ± 1.61	90.51 ± 8.78	**62.47 ± 10.35**	**152.98 ± 16.13**	18.07 ± 2.86	190.02 ± 15.79	1.48 ± 0.22
Adult × June × 2013	16.38 ± 3.19	72.39 ± 8.66	**51.31 ± 12.88**	**124.07 ± 16.56**	10.75 ± 2.56	161.59 ± 19.69	1.50 ± 0.46
Adult × September × 2011	18.04 ± 1.15	97.82 ± 5.37	**82.29 ± 7.35**	**180.12 ± 9.36**	17.12 ± 1.45	215.28 ± 9.15	1.19 ± 0.13
Adult × September × 2013	19.18 ± 3.57	70.91 ± 9.28	**54.12 ± 13.77**	**129.32 ± 16.37**	11.25 ± 2.92	166.70 ± 21.07	1.39 ± 0.39
Probability levels							
Age (A)	n.s.	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**	n.s.
Season (S)	n.s.	n.s.	**0.000**	**0.007**	n.s.	n.s.	**0.010**
Year (Y)	n.s.	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
A × S	**0.043**	**0.008**	n.s.	**0.006**	n.s.	**0.004**	n.s.
A × Y	n.s.	**0.005**	n.s.	n.s.	**0.003**	n.s.	n.s.
S × Y	**0.006**	n.s.	**0.035**	n.s.	n.s.	n.s.	n.s.
A × S × Y	n.s.	n.s.	**0.048**	**0.028**	n.s.	n.s.	n.s.

are more adapted. Furthermore, higher values of palisade parenchyma combined with higher leaf blade have been recorded under stress conditions, and will facilitate the uptake of CO_2 and hence photosynthetic activity under drought conditions (Guerfel et al., 2009), a behavior detected for leaves collected in 2011, a year with a lower amount of rainfall.

3.3. Leaf vessel dimensions

Vessel dimensions were significantly affected by age and season factors in different ways (Table 3). Leaf age influenced phloem and xylem thickness and vascular bundle width, with higher values recorded for old leaves, while young leaves presented higher values for vessel area and vessel perimeter. Although little information about these characteristics in *Salvia officinalis* is available, there are some reports concerning other *Salvia* species, specifying similar dimensions (Kowalczuk et al., 2014) for vessels. Seasonal variations were also found in several of the vessel dimensions analyzed (Table 3), with higher values found in June for vessel area, perimeter, and vascular bundle width. The interaction of those two factors (age and season) also resulted in significant variation in vessel area and perimeter, as well as in phloem thickness and xylem/phloem ratio. Vessel dimensions are linked to several parameters related to water transport safety, like the vulnerability index or relative hydraulic conductivity, and, ultimately, can influence the potential for carbon uptake (Zimmermann, 1983).

3.4. Leaf gas exchange and chlorophyll a fluorescence

Considering results for leaf gas exchange (Table 4), no significant variation was caused by the studied factors (in this situation, only leaf age and month of harvest) for photosynthetic rate (A). This lack of variation in A can partially justify the similar water status also recorded for sage in the present work, as several works point out the relationship between those two parameters (e.g., Chaves et al., 2003). For stomatal conductance (g_s), significant variations were observed, caused by the season factor, with higher values recorded in June compared to September. Lower values of g_s have been correlated to lower leaf water potential or to higher vapor pressure deficit (VPD) (Lambrecht et al., 2011), but also to increasing temperatures (Damour et al., 2010). The recorded decrease in g_s in September is likely related to the higher temperatures recorded in this month and higher VPD, although it should be pointed out that this month recorded higher rainfall, which may indicate that other factors affected this specific parameter. Intrinsic water-use efficiency (A/g_s) also displayed significant differences between June and September, directly linked to the variations in g_s in those months. Reduction in stomatal conductance has been referred to as the first defense mechanism to maintain plant water status (Rogiers et al., 2011), usually leading to an increase in A/g_s, as detected in the present work, but also to a decrease in transpiration rate (E), which was not detected by us. In fact, significant variations were found in E, caused by the month,

Table 3. Values (mean ± standard deviation) for traits of conductive vessels of *Salvia officinalis* leaves collected in 2013 and probability levels of the effects of age and season as determined by ANOVA. ns: not significant; na: results not available. In bold, results shown to be affected by the studied factors and/or their interaction.

	Vascular bundle width (µm)	Vessel area (µm²)	Vessel perimeter (µm)	Phloem thickness (µm)	Xylem thickness (µm)	Xylem phloem ratio
Age (A)						
Young	**312.48 ± 59.34**	**103.45 ± 24.06**	**37.37 ± 4.62**	**43.01 ± 13.24**	**117.04 ± 14.61**	2.89 ± 0.75
Adult	**372.71 ± 96.12**	**83.03 ± 34.39**	**32.39 ± 6.08**	**53.77 ± 32.39**	**152.48 ± 43.98**	3.14 ± 0.79
Season (S)						
June	**357.36 ± 100.28**	**109.26 ± 27.69**	**37.35 ± 4.86**	49.91 ± 33.76	127.89 ± 44.79	2.89 ± 0.82
September	**324.31 ± 64.26**	**84.68 ± 28.32**	**33.81 ± 6.01**	45.91 ± 13.04	134.93 ± 26.53	3.06 ± 0.73
A × S						
Young × June	333.59 ± 64.93	**103.51 ± 27.28**	**36.66 ± 5.29**	**40.84 ± 8.41**	115.96 ± 14.72	**2.97 ± 0.78**
Young × September	294.18 ± 47.93	**103.39 ± 21.37**	**37.98 ± 3.94**	**44.89 ± 16.23**	117.98 ± 14.69	**2.81 ± 0.73**
Adult × June	401.50 ± 137.23	**119.94 ± 26.10**	**38.62 ± 3.76**	**66.76 ± 53.04**	150.03 ± 69.22	**2.75 ± 0.90**
Adult × September	357.78 ± 64.18	**63.89 ± 19.10**	**29.17 ± 4.26**	**47.04 ± 8.37**	153.76 ± 23.98	**3.34 ± 0.65**
F values and probability levels						
Age (A)	**0.000**	**0.021**	**0.000**	**0.004**	**0.000**	n.s.
Season (S)	**0.011**	**0.000**	**0.000**	n.s.	n.s.	n.s.
A × S	n.s.	**0.000**	**0.000**	**0.015**	n.s.	**0.022**

Table 4. Values (mean ± standard deviation) for leaf gas exchange parameters of *Salvia officinalis* leaves sampled in 2011 and probability levels of the effects of age and season as determined by ANOVA. ns: not significant; na: results not available. In bold, results shown to be affected by the studied factors and/or their interaction.

	A (μmol $m^{-2}s^{-1}$)	g_s (mmol $m^{-2}s^{-1}$)	A/g_s (μmol mol^{-1})	C_i (ppm)	E (mmol $m^{-2}s^{-1}$)
Age (A)					
Young	18.89 ± 3.06	266.54 ± 129.96	90.82 ± 49.52	190.06 ± 79.47	**3.54 ± 1.59**
Adult	20.62 ± 1.75	294.85 ± 196.65	104.35 ± 64.87	169.04 ± 99.43	**2.66 ± 0.79**
Season (S)					
June	19.43 ± 2.96	**351.85 ± 198.31**	**81.10 ± 54.99**	201.24 ± 85.88	**2.15 ± 0.66**
September	20.09 ± 2.25	**209.54 ± 76.56**	**114.06 ± 56.16**	157.85 ± 89.77	**4.05 ± 1.10**
A × S					
Young × June	18.21 ± 3.18	**258.82 ± 188.28**	**109.57 ± 65.19**	**155.92 ± 100.34**	**2.12 ± 0.88**
Young × September	19.57 ± 2.98	**274.27 ± 24.59**	**72.06 ± 14.18**	**224.20 ± 28.29**	**4.96 ± 0.25**
Adult × June	20.65 ± 2.29	**444.89 ± 170.41**	**52.64 ± 19.47**	**246.57 ± 32.23**	**2.18 ± 0.39**
Adult × September	20.60 ± 1.16	**144.81 ± 48.76**	**156.06 ± 50.26**	**91.50 ± 80.03**	**3.14 ± 0.81**
F values and probability levels					
Age (A)	n.s.	n.s.	n.s.	n.s.	**0.001**
Season (S)	n.s.	**0.004**	**0.038**	n.s.	**0.000**
A × M	n.s.	**0.002**	**0.000**	**0.000**	**0.000**

not following this previous assumption, considering that we detected higher values in September rather than June. Transpiration rates are linked to environmental factors such as temperature, VPD, or radiation (Sánchez-Díaz and Aquirreola, 2000). The higher temperatures recorded in September may have led to an increase in this parameter, due to increased water vapor pressure inside the leaf. The reduction in E in adult leaves follows a trend already observed in many other species, which occurs until full expansion of the leaf, from which point onwards a reduction could be expected (Wang et al., 2014). The interaction of both factors resulted in significant variations in g_s, A/g_s, C_i, and E. When analyzing leaf gas exchange variations, the effect that the internal architecture of the leaves can have on some of the parameters should also be taken into consideration (Niinemets et al., 2012), which can also be responsible, in addition to environmental factors, for the recorded data. Several important correlations between leaf morpho-anatomical traits and leaf gas exchange were found, but with differences depending on leaf age. When performing regression analysis and keeping only those in which more than 50% of the variation in the dependent variable can be explained by the independent variable ($R^2 > 0.5$), for young leaves, several significant correlations were found between leaf tissue thickness and leaf gas exchange parameters (Table 5). In fact, upper epidermis + cuticle thickness was negatively correlated with both g_s and C_i, with a positive correlation being found with A/g_s. Furthermore, A was also negatively correlated with leaf blade thickness. For adult leaves (Table 5), significant correlations were found between A/g_s and spongy parenchyma, between leaf area and C_i, g_s, and A/g_s (negative correlation), and between leaf succulence and C_i (negative correlation) and A/g_s. Interestingly, none of the correlations found were detected in both young and adult leaves, suggesting different associations between morpho-anatomical traits and leaf gas exchange, dependent on leaf developmental stage.

For chlorophyll a fluorescence parameters (Table 6), significant variations caused by leaf age and month of harvesting were recorded for maximum photochemical efficiency of PSII (F_v/F_m) and for nonphotochemical quenching (NPQ). For F_v/F_m, the recorded values are near the optimal of 0.83 (Maxwell and Johnson, 2000), indicating high photosynthetic performance. The reduction detected in June may have been due to a protective measure used in order to shield the photosystems from oxidation (Baker, 2008), due to high irradiance reaching the leaves during that month. This is further confirmed by the higher values recorded in this month of NPQ, a mechanism used by plants to dissipate excess energy as heat (Maxwell and Johnson, 2000). The interaction of both factors only resulted in significant effects in NPQ.

As sage is used industrially for different food, cosmetic, and pharmaceutical preparations (Pellegrini et al., 2015) the influence of morpho-anatomical parameters in processing cannot be overlooked. Water content is critical when processing includes the drying of several aromatic plants, including sage (Kouhila et al., 2001). In

Table 5. Regression analysis of leaf tissue thickness and leaf gas exchange parameters (only those with $R^2 > 0.5$ presented).

	Dependent–Independent	Linear Regression	R^2	P
Young leaves	g_s – Upper epidermis + cuticle	y = –37.501x + 980.02	0.6716	0.000
	C_i – Upper epidermis + cuticle	y = –21.118x + 591.85	0.5695	0.001
	A/g_s – Upper epidermis + cuticle	y = 13.815x – 172.03	0.6276	0.000
	A – Leaf blade	y = –0.1185x + 39.521	0.5526	0.001
	F_v/F_m – E	y = 0.0143x + 0.81	0.5149	0.002
	NPQ – E	y = 0.1005x + 0.2709	0.7803	0.000
Adult leaves	A/g_s – Spongy parenchyma	y = 3.0359x – 108.02	0.5033	0.002
	C_i – Leaf area	y = 12.309x – 58.949	0.6780	0.001
	g_s – Leaf area	y = 29.569x – 246.98	0.7262	0.000
	A/g_s – Leaf area	y = –9.2827x + 284.26	0.6808	0.001
	C_i – Succulence	y = –2233.3x + 790.93	0.5571	0.005
	A/g_s – Succulence	y = 1726.3x – 367.73	0.5844	0.004

Table 6. Values (mean ± standard deviation) for chlorophyll a fluorescence of *Salvia officinalis* leaves sampled in 2011 and probability levels of the effects of age and season as determined by ANOVA. ns: not significant; na: results not available. In bold, results shown to be affected by the studied factors and/or their interaction.

	F_v/F_m	qP	Φ_{PSII}	ETR	NPQ
Age (A)					
Young	**0.86 ± 0.03**	0.71 ± 0.11	0.48 ± 0.08	304.78 ± 52.45	**0.59 ± 0.23**
Adult	**0.84 ± 0.04**	0.69 ± 0.16	0.42 ± 0.10	265.46 ± 66.05	**0.91 ± 0.10**
Season (S)					
June	**0.83 ± 0.02**	0.72 ± 0.13	0.45 ± 0.09	284.74 ± 62.56	**0.81 ± 0.08**
September	**0.87 ± 0.04**	0.68 ± 0.12	0.45 ± 0.10	285.50 ± 63.44	**0.69 ± 0.31**
A × S					
Young – June	0.84 ± 0.02	0.76 ± 0.12	0.49 ± 0.10	312.09 ± 65.50	**0.78 ± 0.06**
Young – September	0.88 ± 0.03	0.67 ± 0.08	0.47 ± 0.06	297.48 ± 38.50	**0.41 ± 0.18**
Adult – June	0.82 ± 0.02	0.68 ± 0.13	0.41 ± 0.08	257.39 ± 48.86	**0.84 ± 0.10**
Adult – September	0.85 ± 0.05	0.69 ± 0.16	0.43 ± 0.13	273.53 ± 82.54	**0.96 ± 0.06**
F values and probability levels					
Age (A)	**0.043**	n.s.	n.s.	n.s.	**0.000**
Season (S)	**0.002**	n.s.	n.s.	n.s.	**0.000**
A – M	n.s.	n.s.	n.s.	n.s.	**0.004**

this circumstance, these works point out the fact that the initial moisture content can influence the drying rate. This may result in the need to use more time/temperature to reach the optimum drying conditions, regarding storage and further processing, but that can result in losses in the quality of the product (Tanko et al., 2005). Another important parameter regarding the drying of sage is the leaf area. A higher area results in an increased surface for water loss, which in turn favors quick drying (Tanko et al., 2005). Essential oil content and composition in plant are related to several factors (Sangwan et al., 2001), some of them correlated to leaf traits. Of those, photosynthesis is one factor that can be more directly connected to leaf traits (Niinemets, 1999), ultimately affecting one of the most important characteristics of sage, its essential oil content and composition.

The results of the present work allow a thorough characterization of *Salvia officinalis* L. var. *purpurascens* in several parameters, providing new information about this plant. In addition to this characterization, morpho-anatomical variations were found caused by the developmental stage, season, or year of harvest. However, more importantly, key parameters that will affect leaf composition, namely leaf gas exchange and chlorophyll a fluorescence, were also affected by leaf age and season, indicating that these factors should be considered when *Salvia officinalis* L. var. *purpurascens* plants are to be included in cultivation systems, aiming for the production of high quality commodities. Furthermore, the results show the great developmental changes occurring in the leaves of *Salvia officinalis* L. var. *purpurascens* but also how they respond and adapt to changes occurring in environmental conditions.

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