



Pollen Ole e 1 content variations in olive cultivars of different Portugal regions

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Abstract Olive is recognized as a crop with great impact in agricultural, socioeconomic, environmental and public health sectors. The last is becoming more important during recent years as consequence of the increase of the pollen allergy in south Europe prompted by the widespread *Olea* pollen allergic reactions. The aim of the study was to quantify, for the first time, the variations of the Ole e 1 allergen amount in *Olea* pollen grains from four cultivars in three regions of Portugal. How weather parameters can affect the allergen production was also assessed. The study was conducted in three olive producer areas of Portugal from 2010 to 2013, Santarém (Central), Elvas (Southeast) and Mirandela (Trás-os-Montes region, Northeast). Mature pollen of four different cultivars (Cobrançosa, Arbequina, Picual and Verdeal) was

collected during the olive flowering season. Ole e 1 was quantified using specific 2-site antibody ELISA. Pollen of the olive groves at the boundary *Olea* bioclimatic distribution in the Mirandela registered the higher allergen content for all varieties in each study year. Arbequina was the variety that showed the lower Ole e 1 allergen concentration, whereas the higher content was registered for Cobrançosa. The main meteorological parameters that influenced the allergen Ole e 1 concentration in the pollen grains were the rainfall and temperatures related variables. The knowledge of the allergenicity in different olive cultivars is an important tool in the selection of the most adequate for planting as ornamental crop and to adjust the pollen extracts used for diagnosis or even immunotherapy.

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1 Introduction

The olive (*Olea europaea* L.) crop has been cultivated around the world for several centuries. Since Roman times (~ 2000 cal yr BP) olive trees and olive oil production have represented a significant agricultural resource, with a significant expansion along the Mediterranean basin countries (Martín-Puertas et al. 2008). Nowadays, its cultivation goes beyond the

south Europe and north Africa, to the south-western Asia and in America, including California, Chile and Argentina (Barranco et al. 2008).

Olive is recognized as a crop with a great impact in the agricultural, socioeconomic, environmental and public health sectors. The last is becoming more and more important in recent years as consequence of the increase of the pollen allergy rates in south Europe prompted by the widespread *Olea* pollen allergic reactions (D'Amato et al. 2007; Barber et al. 2008). The increase of the area dedicated to the olive groves and the proliferation of the *Olea* trees for ornamental purposes can contribute to the increase of the airborne pollen concentration during the olive flowering season. The olive industry is one of the major economic activities in the south European countries (Galán et al. 2005), particularly in Portugal (Sánchez-Mesa et al. 2005). In Portugal, the total extend of planted olives is around 360 thousand hectares, mainly concentrated in the Alentejo region but also with important plantations in the Trás-os-Montes region (INE 2018).

Sensitization rates to olive pollen in Portugal reaches 64.7% of allergenic patients (Camacho 2017). Fifteen allergens have been identified and characterized in olive pollen extracts, from Ole e 1 to Ole e 15 (Villalba et al. 2014; Oeo-Santos et al. 2018; San Segundo-Acosta et al. 2019). The major allergen from olive tree pollen is Ole e 1 (Asturias et al. 1997; Villalba et al. 1993). This protein is account with two variants, the first glycosylated (80% to 85% of the total allergen) with a molecular mass 20 kDa and the second non-glycosylated with a molecular mass of 18.5 kDa (Asturias et al. 1997, Villalba et al. 1993). Both variants showed a high degree of polymorphism and can promote the allergenicity of the molecule (Van Ree et al. 2000). For this reason, around of the 80% sensitized people to olive pollen shows allergenic symptoms to Ole e 1 (Villalba et al. 2014). Ole e 1 is the best-characterized allergen from olive, and their expression occurs only in pollen grains but not in the olive fruit. In addition, Ole e 1 is involved in the pollen hydration and/or germination process by homology to the cysteine-rich extracellular protein LAT52 (Tang et al. 2002). Therefore, when the pollen is not properly hydrated, it is not capable of germinating, drastically weakening fertilization process which prompts a reduction of olive production. During the flowering stage, changes in the temperature can induce a reduction of the pollen germination rate slowing down

fertilization. In addition, the optimum temperature for pollen germination can vary between cultivars of the same species (Vuletin Selak et al. 2019). During spring, when *Olea* flowering occurs, significant changes in the daily temperature were observed, altering the reproductive process (Vuletin Selak et al. 2019).

The aim of the present study was to quantify, for first time, the variations of the Ole e 1 allergen amount in *Olea* pollen from four cultivars in three regions of Portugal. How weather parameters might affect the allergen production was also assessed. This information will help to identify suitable cultivars for planting in future olive regions in order to obtain cultivars that combine optimal production of olives and pollen with lower allergen content.

2 Materials and methods

The field study was conducted in three important olive produced regions of Portugal from 2010 to 2013, Santarém in the Central, Elvas in the Alentejo region (southeast) and Mirandela in the Trás-os-Montes region (Northeast) (Fig. 1). Mature pollen of three different cultivars (Cobrançosa, Arbequina and Picual) in Santarém and Elvas regions and three (Cobrançosa, Arbequina and Verdeal) in Mirandela region was collected during the olive flowering season during the month of May in experimental fields. Flowering season was determined by phenological observations and every year the sampling date occurred at the full flowering stage, where at least 50% of flowers are open. During 2011 and 2012, no data were obtained for Mirandela and Elvas regions respectively. For each cultivar, four trees were randomly chosen and pollen samples were collected from 15 inflorescences per tree, one per each canopy quadrant, selected at random in the middle position of fruiting shoots in order to obtain a representative composite sample per each cultivar. Pollen grains were collected into small paper boxes (one separate box per each cultivar), transported to the laboratory and dried overnight at 24 °C. Afterwards, the pollen was released from the anthers by passing through different grades of sieves to obtain pure pollen, and stored at − 20 °C until analysis.

To obtain crude protein extracts we suspended 10 mg of pollen grains for each cultivar in 1 mL of phosphate-buffered saline (pH 7.4). Soluble proteins were extracted

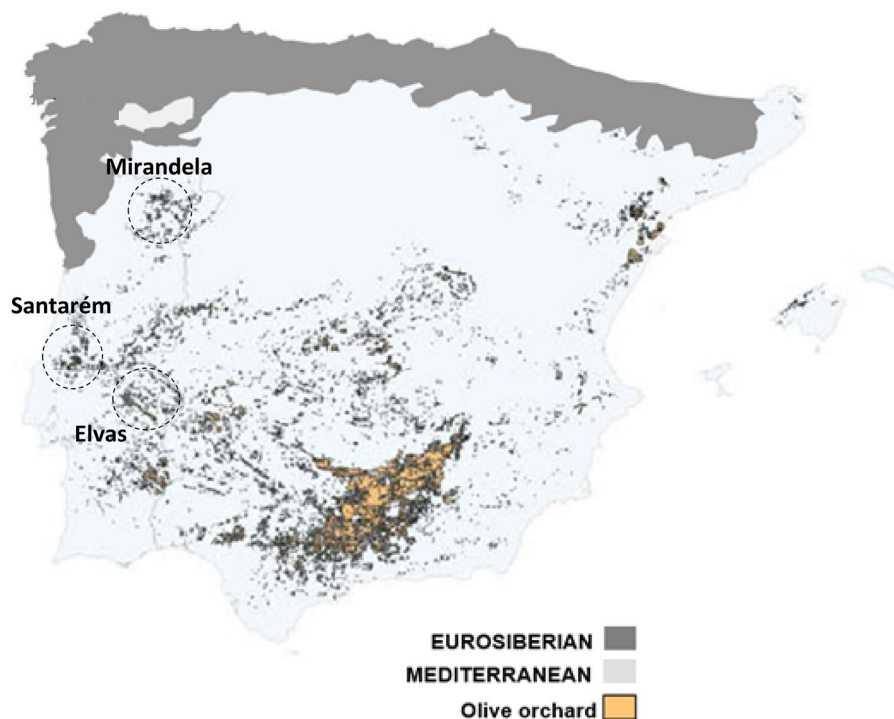


Fig. 1 *Olea* distribution in the main bioclimatical Regions of the Iberian Peninsula, and location of the studied olives groves

by continuous stirring during 3 h at 4 °C. The suspension was centrifuged at 13,200 rpm for 30 min at 4 °C. The supernatant was stored at -20 °C.

Ole e 1 concentration in the different samples was quantified by specific 2-site antibody ELISA using an Ole e 1 antibody purified from *O. europaea* pollen by standard chromatographic methods (Arilla et al. 2002). Microtiter plates were coated with Ole e 1 monoclonal antibody (0.55 mg/mL) (Roxal Industrial Farmacéutica, Spain) over night at 4 °C. The plates were then blocked with PBS (pH 7.4), containing 1% bovine serum albumin and 0.05% Tween 20. Next, the plates were incubated with natural antibody allergens starting from a 2.5 µg/mL stock of natural Ole e 1. Tenfold serial dilutions were performed in PBS-BSA-T, containing 50–0 ng/mL. At the same time, we added 100 µL/well of the extracted pollen samples. Then, secondary biotinylated antibody (0.72 µg/µL) (Roxal Industrial Farmacéutica, Spain) and 0.25 µg/mL streptavidin-peroxidase (Sigma) were added. All incubations were performed for 1 h at 37 °C. Three washes with PBS-T between successive steps were conducted. Finally, the plates were incubated at room temperature in the dark with o-phenylenediamine (OPD) (Sigma), and the reaction stopped at 30 min

with H₂SO₄ (3 M). Finally, the absorbance was measured at 492 nm. The extracts were assayed as dilution series, and the sample concentrations were interpolated from the linear portion of the standard curve in µg/mL of sample.

The most important meteorological variables (maximum, minimum and average temperatures, relative humidity and rainfall) were continuously monitored during the previous months to flowering by complete meteorological stations in the sampled regions Santarém, Elvas and Mirandela.

Regarding the statistical analysis of the results, average and standard deviation of allergen concentration were determined per cultivar/year/location, which presented a normal distribution tested using a Shapiro–Wilk test. To study the main effect of cultivar, year and location in the pollen Ole e 1 allergen content variations, a general linear model (GLM) procedure was applied by using the main effects ANOVA analysis. The variable cultivar was used as fixed factor and the variables years and location as random factors since they represent a random subset of a larger population available. The Tukey's HSD post hoc test (p value = 0.05) was used to perform multiple comparisons among each effect. A

principal component analysis (PCA) was performed to detect any structure in the relationships between variables (Ole e 1 and weather parameters), and to classify variables according to their influence on dependent variables' variation (Tipping and Bishop 1999).

All statistical analysis was performed using the STATISTICA software v13.

3 Results

The average of the total allergen concentration in the available years for the studied cultivars (Cobrançosa,

Arbequina, Picual and Verdeal) showed that the region with the higher amount of Ole e 1 allergen in the olive pollen was Mirandela following by Santarém, and finally Elvas (Fig. 2a). In addition, when the total allergen concentration in each year was analysed we observed that during 2013, Mirandela registered the higher allergen amount with 655 $\mu\text{g}/\text{mL}$. By the contrary, Santarém was the area that in 2011 presented the lowest allergen content in the pollen grains with 231 $\mu\text{g}/\text{mL}$ (Fig. 2b).

The cultivar with higher allergen concentration in the pollen grains in Santarém was Cobrançosa during 2010 with (179 $\mu\text{g}/\text{mL}$) whereas the cultivar with lower Ole e 1 content was Arbequina (43 $\mu\text{g}/\text{mL}$) in

Fig. 2 Total pollen Ole e 1 allergen amount **a** during the study years in all study cultivars, **b** in each study year and in each study area

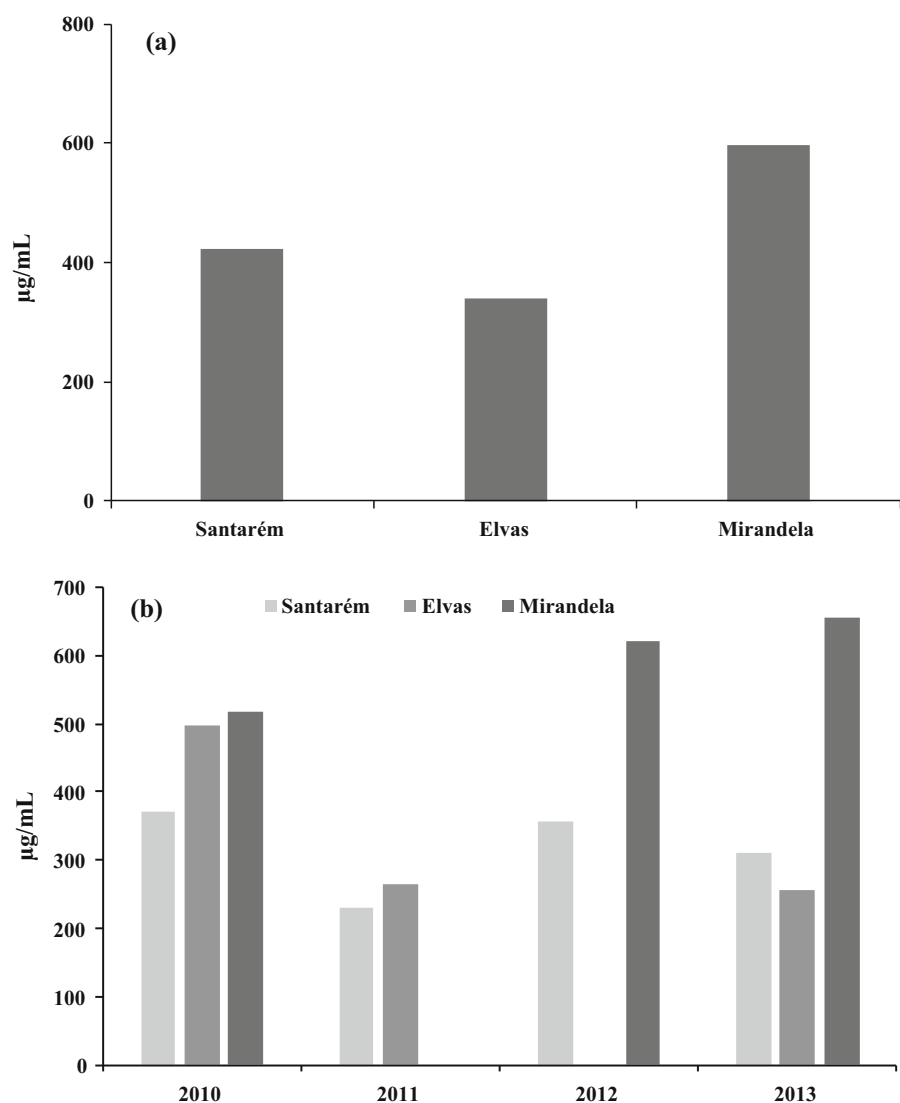
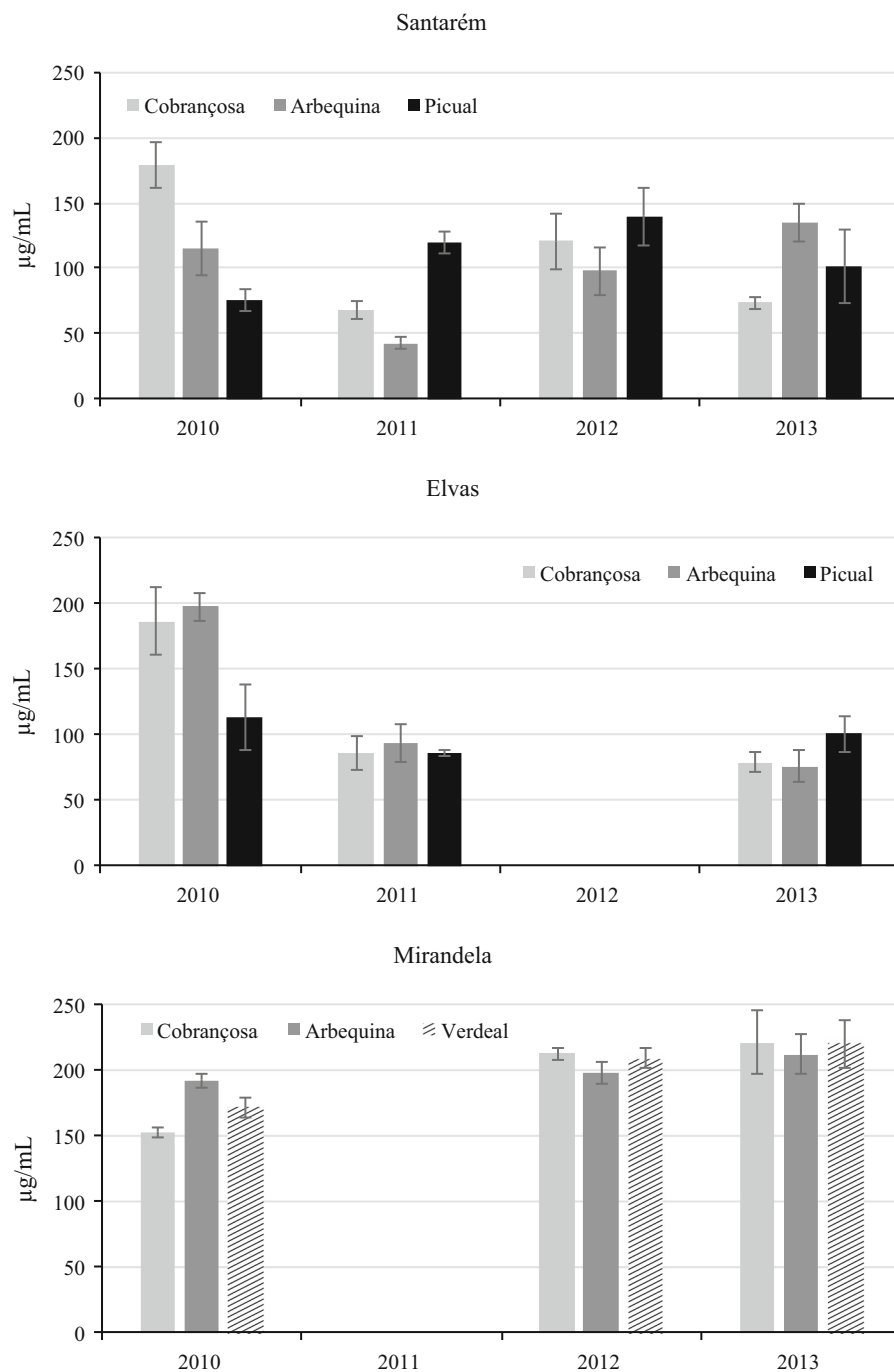


Fig. 3 Pollen Ole e 1 content in each study area and in each cultivar during the study years



2011 (Fig. 2). In Elvas the cultivar with higher amount of allergens was Arbequina with 198 µg/mL in 2010. The same cultivar showed the lowest allergen concentration during the studied period with 76 µg/mL in 2013. Finally, the cultivars with the higher Ole e 1 concentration in Mirandela were Cobrançosa and

Verdeal during 2013 with 221 µg/mL in each, whereas the lowest allergen concentration in the pollen grains was registered for Cobrançosa during 2010 with 153 µg/mL (Fig. 3).

A General Linear Model (GLM) was conducted in order to ascertain if the studied cultivars, years and

Table 1 General Linear Model (GLM) results obtained using a main effects ANOVA analysis between the content of Ole e 1 and the cultivars studied (fixed factor), the years and the location (random factors) of the study area

	Ole e 1 allergen				
	Type III sum of squares	df	Mean square	F	Sig.
Intercept	1,254,308	1	1,254,308	32.892	0.015
Cultivar	518	3	173	0.139	0.936
Year	23,353	3	7784	6.304	0.001
Location	69,405	2	34,702	28.103	0.000
Error	100,022	81	1235	–	–

Table 2 Homogeneous groups formed considering the Ole e 1 allergen content per cultivar, year and location after the pairwise comparisons using the Tukey's HSD post hoc test (p value = 0.05)

Ole e 1 ($\mu\text{g/mL}$)				
Cultivar	Mean allergen	Groups		
		1	2	3
Cobrançosa	138.05	x		
Arbequina	135.99	x		
Picual	105.26		x	
Verdeal	200.58			x
Year				
2010	153.79	x		
2011	82.88		x	
2012	162.97	x		
2013	135.53	x		
Location				
Santarém	105.74	x		
Elvas	113.17	x		
Mirandela	199.07		x	

location had statistical significant impact in the Ole e 1 content variations. It was observed that allergen variations were significantly influenced by the year ($p < 0.01$) and location ($p < 0.000$) (Table 1). Concerning the multiple comparisons among each factor, it was observed significant differences in the average Ole e 1 allergen content between Verdeal and all other cultivars and between Picual and all other cultivars. Arbequina and Cobrançosa presented similar average Ole e 1 content. Concerning the random effects location and year, it was observed that Ole e 1 allergen content was significantly different between Mirandela

and the other two locations (in Mirandela, the pollen presented higher Ole e 1 content) and different between the year 2011 and all the others, which did not present significant differences among them (Table 2).

When the behaviour of the weather variables was analysed in the Santarém region the higher average maximum, minimum and mean temperature was registered during 2011 as well as the higher relative humidity, and the lower amount of rainfall in 2013 (Fig. 4). In Mirandela was observed higher average rainfall in 2010, the lower average minimum temperature and relative humidity in 2012, and the lower average maximum and mean temperatures take place in 2013. Finally, the lower protein content was registered in 2011 in Santarém coinciding with the peak of maximum, minimum and average temperatures during this year (Fig. 4).

In order to know the possible correlation between the main weather variables and the Ole e 1 concentration, a PCA analysis was applied. In the present study 6 principal components (PC) have been extracted, since they had eigenvalues greater than or equal to 1.0 and they account together for 94.7% of the variability in the original data (Table 3). In general, the allergen content loads in the fifth PC, and it is correlated with minimum temperature of February and April, and rainfall of November and January. In order to have a visual comprehension of the relationship between the Ole e 1 and weather variables, a plot with PC1 versus PC2 is presented. The results of the PCA analysis showed a high positive degree of association between the daily Ole e 1 allergen levels and average and minimum temperature and rainfall (Fig. 5).

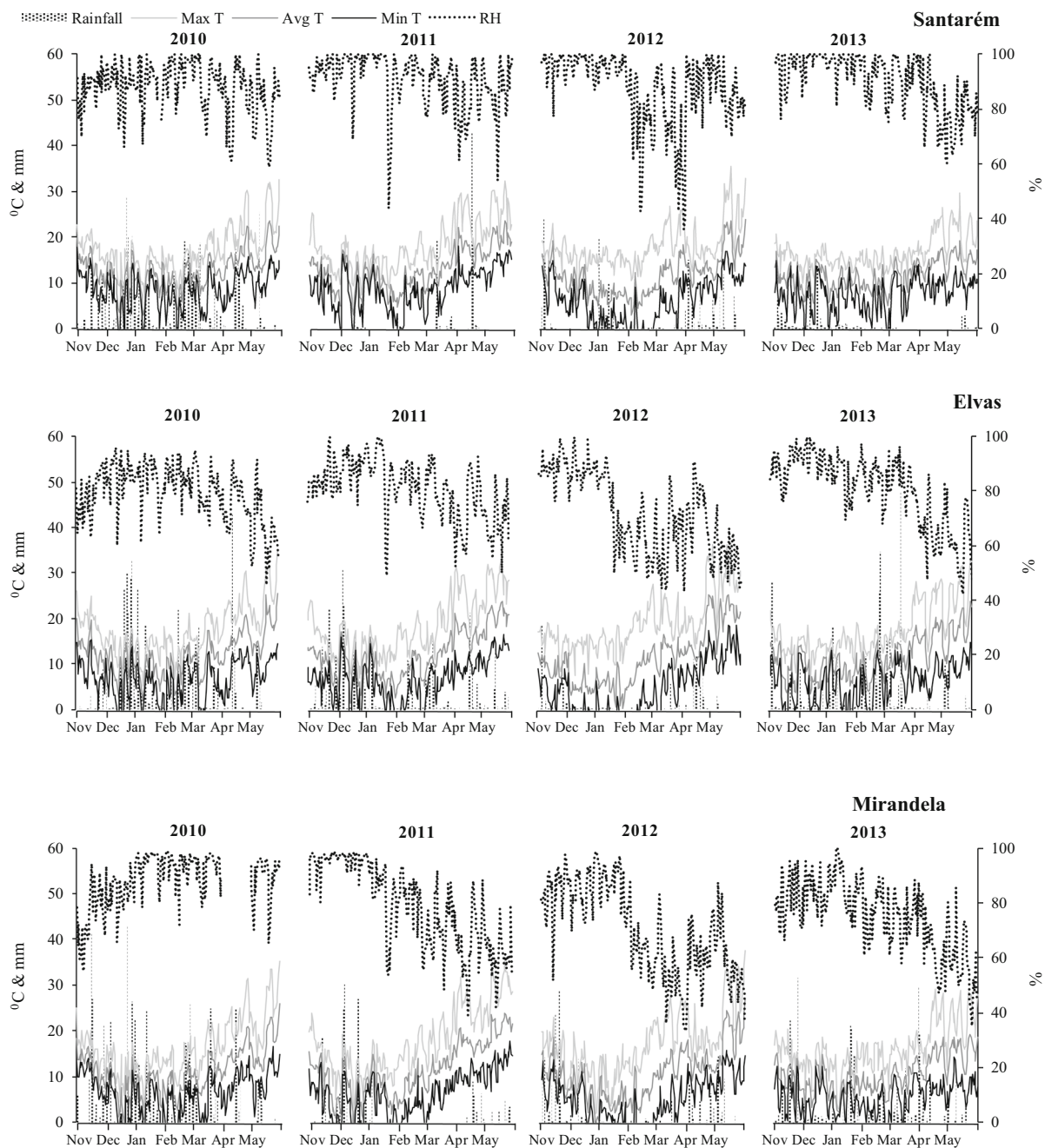


Fig. 4 Meteorological variables during the study period (from 2010 to 2013) in each study area

4 Discussion

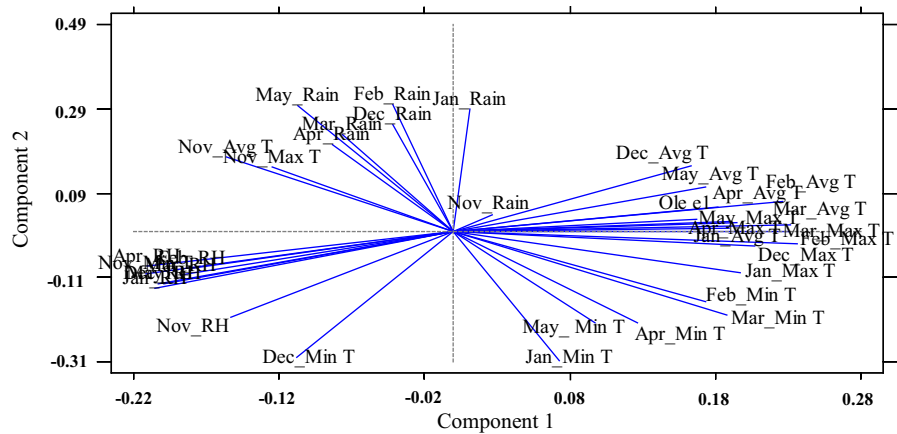
According to the EUROSTAT, in the European Union, olive groves cover around 4.6 Mha, with around 2.5 Mha being the surface of the olive grove in the Iberian Peninsula (AAO 2011). As consequence of their great

extension, the olive pollen allergens are currently considered as one of the most important cause of respiratory allergies in the southern European countries (D'Amato et al. 2007). In the present study, the Ole e 1 content in the pollen grains of four *Olea* cultivars in three different areas was assessed during a

Table 3 Dimension weights of principal component analyses performed with the weather parameters and Ole e 1 content (values in bold present $p < 0.05$)

Components	1	2	3	4	5	6
<i>Eigenvalue</i>	17.287	7.156	3.783	2.101	2.029	1.738
<i>Per cent of variance</i>	48.020	19.876	10.507	5.837	5.636	4.826
<i>Cumulative percentage</i>	48.020	67.896	78.403	84.240	89.876	94.703
Ole e 1	0.167	0.027	0.130	− 0.006	0.290	− 0.184
November						
MaximumT	− 0.124	0.151	0.030	0.452	0.242	0.010
MinimumT	− 0.211	− 0.102	− 0.030	0.053	0.250	− 0.088
AverageT	− 0.156	0.176	− 0.125	0.229	0.055	− 0.286
Relative Humidity	− 0.153	− 0.203	− 0.211	− 0.190	− 0.084	− 0.128
Rain	0.027	0.039	− 0.338	− 0.395	0.147	− 0.076
December						
MaximumT	0.207	− 0.035	0.102	0.225	0.020	0.169
MinimumT	− 0.108	− 0.298	0.103	− 0.013	0.202	0.017
AverageT	0.163	0.156	− 0.035	0.252	− 0.178	− 0.206
Relative Humidity	− 0.203	− 0.123	− 0.132	− 0.183	− 0.069	− 0.096
Rain	− 0.042	0.251	− 0.051	− 0.105	0.422	− 0.185
January						
MaximumT	0.197	− 0.099	− 0.047	− 0.273	0.095	− 0.189
MinimumT	0.072	− 0.305	0.011	0.075	0.319	− 0.014
AverageT	0.189	0.022	− 0.119	− 0.100	− 0.021	− 0.374
Relative Humidity	− 0.205	− 0.136	− 0.157	0.065	− 0.038	0.036
Rain	0.011	0.288	− 0.118	− 0.040	0.361	0.116
February						
MaximumT	0.236	− 0.029	0.003	0.013	− 0.007	− 0.085
MinimumT	0.173	− 0.167	0.065	0.026	0.284	0.139
AverageT	0.226	0.071	− 0.040	0.034	− 0.048	− 0.168
Relative Humidity	− 0.180	− 0.089	− 0.306	0.037	0.030	− 0.002
Rain	− 0.042	0.300	− 0.085	− 0.111	0.125	0.293
March						
MaximumT	0.231	− 0.002	0.006	− 0.099	0.066	− 0.140
MinimumT	0.188	− 0.199	0.061	− 0.097	0.174	0.008
AverageT	0.231	0.016	− 0.012	− 0.081	− 0.002	− 0.174
Relative Humidity	− 0.188	− 0.103	− 0.269	0.017	0.000	0.061
Rain	− 0.076	0.229	− 0.198	− 0.184	− 0.059	0.248
April						
MaximumT	0.189	0.006	− 0.292	0.075	− 0.021	0.150
MinimumT	0.126	− 0.216	− 0.238	0.183	0.186	0.139
AverageT	0.181	0.058	− 0.307	0.119	− 0.092	− 0.005
Relative Humidity	− 0.211	− 0.082	− 0.093	0.020	0.100	− 0.268
Rain	− 0.083	0.206	− 0.060	0.248	0.099	− 0.244
May						
MaximumT	0.194	0.020	− 0.271	0.076	− 0.012	0.104
MinimumT	0.097	− 0.217	− 0.262	0.183	0.119	0.204
AverageT	0.173	0.104	− 0.297	0.121	− 0.125	− 0.057
Relative Humidity	− 0.199	− 0.124	− 0.073	0.161	− 0.035	− 0.230
Rain	− 0.107	0.296	0.055	− 0.155	0.179	0.106

Fig. 5 Plot of principal components. Factor loadings Factor 1 versus Factor 2. Extraction Principal Components



period of four consecutive study years. The results showed statistically significant differences in the Ole e 1 content between cultivars, years and locations.

GLM results showed discrimination in three homogeneous sub-groups. The first with Cobrançosa and Arbequina, the second one with Picual and the last one with Verdeal cultivar that registered the highest pollen allergen content. These results are according with a previous study investigating the dissimilarity regarding the reactivity of patients' sera, presenting specific IgE levels to *Olea europaea* and the content of Ole e 1 allergen in the pollen extract of twelve Portuguese olive cultivars, where it was observed that the Cobrançosa cultivar induced the most intense bands (Ribeiro et al. 2013). A research conducted in Spain noted the lower Ole e 1 content for the Hojiblanco and Acebuche versus the Picual and Manzanillo cultivars (Fernández-Caldas et al. 2007). Several investigations pointed out the variability of the allergenicity of the pollen grains registered between the different cultivars as a result of the intrinsic genetic acerb of each cultivar (Fernández-Caldas et al. 2007; Hamman-Khalifa et al. 2008). Some authors argued that higher or lower allergenic protein content can be detected for a given pollen grain depending of the plant (Cabrera et al. 2002).

Differences shown by the port-hoc test between cultivars could be due to variations in monitoring conditions, such as year or location. Year to year significant variations were also detected for the different study areas. During 2010 the lower Ole e 1 concentration was registered in the Picual cultivar in Santarém and Elvas, in 2012 the lowest content was observed for Arbequina in Santarém and Mirandela,

and finally in 2013 the cultivar that showed lower Ole e 1 charge was Arbequina in Elvas and Mirandela. All together, the year 2011 was statistically different from all the other.

These results are according with the significant variations detected for the Ole e 1 content in the pollen of six *Olea* cultivars in Spain (Fernández-Caldas et al. 2007). Several investigations agree with this trend, suggesting that the concentration of allergens may vary from year to year and from region to region (Buters et al. 2008, 2012; Galán et al. 2013).

Furthermore, different authors pointed out that bioclimatic condition of the olive grove could highly influence the pollen allergen content, as well as the sensitive of people suffering from pollen allergies to a specific allergen (Casanovas et al. 1997; Morales et al. 2012; Quiralte et al. 2002). Our results showed that the pollen, of the olive groves at the boundary of the *Olea* bioclimatic distribution, in Mirandela northern Portugal registered the highest allergen content for all cultivars. The Ole e 1 protein participates in the pre-germination processes control as well as the pollen tube emergence and guidance (Alche et al. 2004). This reproductive process is affected by weather variables, since the temperature regulates the reproductive development of the olive tree, mainly during pre-flowering and anthesis (Galán et al. 2005; Melo-Abreu et al. 2004; García-Mozo et al. 2009; Sicard et al. 2012). The areas with the higher variations in the optimal temperatures and humidity conditions for a successful olive fertilization were the located at the boundary of its distribution, which could prompt particular stress situations in the trees. In addition, the northern olive groves recorded the lower temperature

regimen along the studies period. Therefore, in response to these signals, the olive tree could increase the expression of the Ole e 1 proteins in these areas in order to ensure the fertilization processes. The knowledge of the microclimatic conditions is crucial for a full understanding of differences in olive reproductive development.

In order to ascertain the effects of the meteorological variables in Ole e 1 concentration in all study areas, a PCA analysis between allergen concentration and weather related variables during the previous months to the flowering was conducted. In general, the principal component analysis highlights the rainfall as the variable correlating the most with allergen concentrations. Ole e 1 content was positively correlated with November and January rainfall and with February and April average Temperature. The rainfall correlation may be related to the availability of water in the soil during the formation of pollen grain and pre-flowering, suggesting less stressful conditions that might affect the expression of Ole e 1.

Considering the temperature influence, it was observed in the work of Buters et al. (2008, 2010) that the Bet v 1 expression only takes place during the flowering stage. However, no significant correlation between meteorological parameters and allergen content were observed for the period corresponding to inflorescence emergence until flowering. The same was observed in our study, with no significant correlation with Ole e 1 content being observed from March to May, corresponding to the equivalent period in *Olea* flower development. In our study only minimum temperature in January and February were significant. Usually during January bud development can be observed, taking place meristematic cell differentiation at the flower buds in order to originate the different inflorescences ramifications. In February it is possible to observe the beginning of differentiation of anther cells (Ribeiro 2008). These results may indicate that weather conditions, particularly in our case, minimum temperature, during the first stages of inflorescence development could be an important indicator in determining the allergen content in a given year. In this sense some authors pointed out annual oscillations in the Ole e 1 concentrations attributed to environmental factors such as temperature and rainfall, even on the previous period to flowering (Fernández-Caldas et al. 2007). Weather parameters, mainly temperature and rainfall are the

main factors affecting the phenology and pollen production of plants (D'Amato et al. 2015).

Previous in vitro studies showed that pollen Ole e 1 allergen expression increases when it germinates into a moisture medium (Alche et al. 2004), the increase of relative humidity induces to the gradual hydration of the pollen grain with the consequent metabolic activation and release of allergens (Fernández-González et al. 2019). Furthermore, meteorological patterns (rainfall and winds) influence the movement and dispersion of allergens in the atmosphere (Dahl et al. 2013). A further investigation could be conducted to ascertain how the potential risk of Ole e 1 allergen load could be determined based on rainfall regime. Temperature represent other important weather variable affecting plant reproductive process, such as pollen tube growth or pollen germination (Vuletin Selak et al. 2019).

During the last years, *Olea* is increasingly planted for ornamental purposes. Therefore, the selection of a cultivar with low allergenicity, considered as a scarce pollen allergen production, is essential in order to prevent allergic reactions in sensitive people. Our results showed the cultivar with the highest Ole e 1 content in the pollen grains was Cobrançosa, whereas the lowest was registered for the Arbequina cultivar. Therefore, this last cultivar could be the most suitable for the use as ornamental tree in public or private gardens, since their allergenic potency could induce lower allergy symptoms in sensitized people. In addition, the knowledge of the allergenicity of the different olive cultivars represents an important tool to adjust the extracts used for diagnosis or even immunotherapy (Alché et al. 2007) highlighting novel perspectives in the epidemiological study of the olive respiratory allergy-related disorders.

5 Conclusions

Significant differences in the olive pollen Ole e 1 content between cultivars, years and regions was detected. The pollen of the olive groves at the boundary *Olea* bioclimatic distribution in Mirandela (northern Portugal) registered the highest allergen content for all cultivars in each study year. By the contrary, the Santarém region showed the lowest total allergen content. Regarding the different cultivars, Arbequina showed the lowest Ole e 1 allergen

concentration, whereas the highest was registered for Cobrançosa. These data indicate that the Arbequina cultivar is the most adequate for planting as ornamental crop, or even for intense cultivation.

The main meteorological parameters that influenced the allergen Ole e 1 concentration in the pollen grains were the rainfall and temperature related variables, depending on the adaptability in each cultivar to the different bioclimatic olive grove area.

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