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Pollen Production of *Quercus* in the North-Western Iberian Peninsula and Airborne Pollen Concentration Trends during the Last 27 Years

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Abstract: Natural forests are considered a reservoir of great biological diversity constituting one of the most important ecosystems in Europe. *Quercus* study is essential to assess ecological conservation of forests, and also of economic importance for different industries. In addition, oak pollen can cause high sensitization rates of respiratory allergies in pollen-allergy sufferers. This study sought to know the pollen production of six oak species in the transitional area between the Eurosiberian and Mediterranean Bioclimatic Regions, and to assess the impact of climate change on airborne oak pollen concentrations. The study was conducted in Ourense (NW Spain) over the 1993–2019 period. A Lanzoni VPPS 2000 volumetric trap monitored airborne pollen. A pollen production study was carried out in ten trees randomly selected in several *Quercus* forest around the Ourense city. Oak pollen represented around 14% of annual total pollen registered in the atmosphere of Ourense, showing an increasing trend during the last decade. Pollen production of the six studied oak species follow the proportions 1:1:2:5:90:276 for *Q. ilex*, *Q. faginea*, *Q. rubra*, *Q. suber*, *Q. pyrenaica*, and *Q. robur* respectively. We detected a significant trend to the increase of the annual maximum temperature, whereas a decrease of the maximum and mean temperatures during three previous months to oak flowering. This could be related with the detected trend to a delay of the oak Main Pollen Season onset of 0.47 days per year. We also found significant trends to an increase of the annual pollen integral of 7.9% pollen grains per year, and the pollen peak concentration of 7.5% pollen grains per year. *Quercus* airborne pollen monitoring as well as the knowledge of the reproductive behavior of the main oak species, bring us an important support tool offering a promising bio-indicator to detect ecological variations induced by climate change.

Keywords: *Quercus*; pollen production; climatic change; Aerobiology

1. Introduction

1.1. Background

Oak is the dominant tree in many of Europe's deciduous forests [1]. Natural oak forests are considered a reservoir of great biological diversity, constituting one of the most important ecosystems in Europe. In Northwestern Spain, the presence of the *Quercus* tree species in forests is an indicator of ecological maturity [2–5], as they are responsible for maintaining the physicochemical and microclimatic characteristics of this ecosystem [4]. The study of oak is not only essential to assess the ecological conservation of forests, but it is of great economic importance to timber and livestock industries [2]. It is also useful in some regions where its fruit is used as nourishment for certain native species of animals, such as pigs [6,7], and for its potential to act as a carbon sink in the future [8].

The last assessment conducted by the Intergovernmental Panel on Climate Change [9] reinforces the findings of temperature increases during the second decade of the 21st century. Each of the last three decades has consecutively been the warmest on instrumental record in terms of the Earth's overall mean surface temperature, which is prompting phenological changes in plants [10]. Tree species have higher levels of genetic diversity compared to other members of the plant kingdom [11], which makes them especially resistant to genetic erosion resulting from climate alterations [12]. Some authors have proposed the use of oak pollen levels as a suitable bio-indicator to detect ecological variations induced by climate change in the Iberian Peninsula [11,12].

1.2. Quercus Ecology

Forests may produce pollen in masses reaching 100 to 1000 Kg/ha [13]. Most of this mass is deposited in lakes and forest floors [14], increasing the productivity of the ecosystem [15,16]. Pollen rapidly decomposes in aquatic and terrestrial ecosystems, releasing high amounts of nutritionally rich matter after deposition [17] as an easily available, digestible, and nutritious food for fungi, bacteria, protozoans, and various groups of invertebrates. Therefore, pollen plays an important role within and across ecosystem nutrient cycling [16].

Quercus is represented by 13 taxa in the Iberian Peninsula [18], being well represented in the Atlantic and Mediterranean regions. In Northwest Spain, the *Quercus* genus is represented by six species [19,20] with *Quercus robur* L. occupying a wider geographical area [19]. These six species can be found in different vegetation series depending on the phyto-geographic sector. In the western area, at an altitude of 700 m, the acidophilic series of *Q. robur* (*Rusco aculeati-Quercetum roboris*) dominates. The stabilized optimum of this vegetation series, with low diversity in tree species, is a dense oak forest accompanied with a certain amount of Pyrenean oak (*Quercus pyrenaica* Wild.) or cork oak (*Quercus suber* L.). At an altitude range of 700 to 1000 m, the dominant species in the more eastern Mediterranean area is *Q. pyrenaica* (*Genisto falcatae-Quercetum pyrenaicae)*, accompanied by *Q. robur* in the Eurosiberian zone although with Mediterranean influence (*Linario triornithophorae-Quercetum pyrenaicae*). The distribution of *Quercus ilex* L. *susp. ballota* is very restricted, showing a residual nature due to the presence of limestone in the Eurosiberian area (*Cephalanthero longifoliae-Quercetum rotundifoliae*) or the presence of a siliceous series in the Mediterranean zone (*Genisto hystricis-Quercetum rotundifoliae)* where it is accompanied by *Quercus faginea* Lam. and *Q. pyrenaica*. One of the most peculiar oak communities in the Mediterranean territory of Northwest Spain is formed by *Quercus suber* L. (*Physospermo cornubiensis-Quercetum suberis*) that occupies a narrow band in the Sil valley near the city of Ourense [19]. *Quercus faginea* Lam. species is only represented by one population in the vicinity of Ourense. Finally, *Quercus rubra* L. is abundantly cultivated as an ornamental species in parks and along avenues of several cities.

1.3. Impact on Human Health

In addition, oak pollen can induce respiratory allergies in many countries [4]. Although it is traditionally assumed that pollen from European *Quercus* species has low allergenicity, some researchers noted the potential responsibility of oak for major cases of allergies in areas with abundant *Quercus* vegetation [21,22]. Previous studies demonstrated that oaks produce a high amount of pollen grains per flower (between 1.9×10^4 and 1.3×10^6 pollen grains) depending on the species [23,24]. Furthermore, symptoms appear frequently over long periods due to flowering overlaps of the different species [4] and their related reactivity with pollen from *Alnus*, *Betula*, *Castanea*, *Olea* and Poaceae [25]. This fact increases their allergenic potential due cross-reaction processes and the so-called "priming effect" [2,7,26]. In Northwest Spain, the sensitization rate to oak pollen among pollen-allergy sufferers, assessed by skin-prick tests, varies between 4% to 12% [27,28]. This high sensitization rate indicates the importance of research in oak pollen allergy [29]. Information about pollen production and airborne dispersion will allow us to enhance the prevention of clinical pollinosis symptomatology and to reduce medicine consumption.

1.4. Goal of the Study

This study plays an important role since it analyzes an abundant taxon in the sampling area whose pollen is considered to be responsible for major cases of allergies. Since pollen masses can increase the productivity of an ecosystem, both terrestrial and aquatic, we studied the pollen production of the most important six oak species in the transitional area between the Eurosiberian and Mediterranean Bioclimatic Regions in Northwest Spain. We also assessed the impact of climate change on the seasonal dynamics of airborne oak pollen.

2. Materials and Methods

2.1. Location and Classification of the Study Area

This study was conducted in the Ourense province, located in a depression at 139 m above sea level in North-western Spain (42°20′ N; 7°52′ W) in a transition zone between the Eurosiberian and Mediterranean Bioclimatic Regions (Figure 1). The climate is oceanic with strong Mediterranean influence. According to the Worldwide Bioclimatic Classification System, Ourense presents a very warm temperature and has low humidity, with an annual mean temperature of 14 °C and annual rainfall around 772 mm [30].

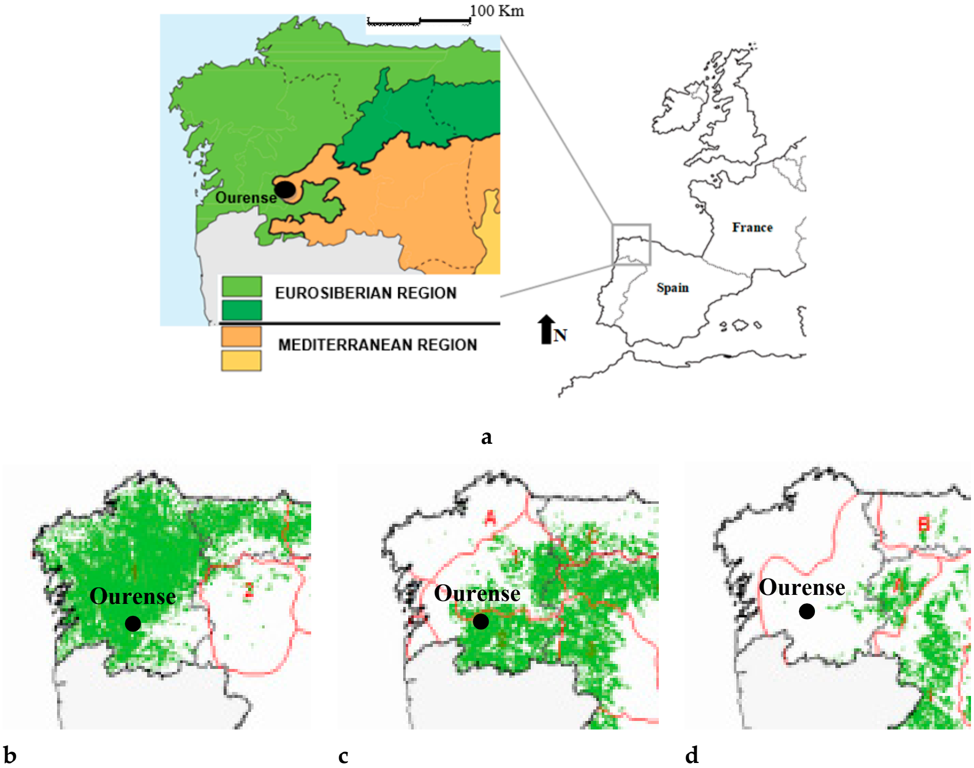

Figure 1. (**a**) Location of the area of study in the North-western of the Iberian Peninsula at the transition zone between the Eurosiberian and Mediterranean Bioclimatic Regions [19] https://www.ign.es/espmap/mapas_ma_eso/MedioESO_Mapa_06.htm. Distribution of the main oak species in the studied area in green: (**b**). *Q. robur*, (**c**). *Q. pyrenaica*, (**d**). *Q. ilex* sub. ballota (http://especiesforestales.com/fichas.html).

2.2. Pollen Production Study

A pollen production study was carried out in several oak forests around the city of Ourense. The selected populations belonged to the main species represented in North-western Spain: *Quercus robur* L., *Quercus pyrenaica* Wild, *Quercus ilex* subsp. *ballota*, *Quercus suber* L., *Quercus faginea* Lam. and *Q. rubra* L. The most prominent forests belongs to *Q. robur* with 61,904 ha and *Q. pyrenaica* with 544,943 ha [31]. In each populations, ten trees were randomly selected to estimate pollen grains per anther, anthers per flower, flowers per catkin, pollen grains per catkin, catkins per tree and pollen grains per tree. The methods developed by Cruden [32] and Hidalgo [33] were used to estimate pollen production per anther in three catkins per tree. Three flowers were selected on each catkin, and three anthers of each flower were sampled. Therefore, pollen production was assessed with 27 anthers per tree (3 catkins × 3 flowers/catkin ×3 anthers/flower). Anthers from fresh flowers close to the anthesis were collected, placed in the bottom of a test tube and 1 mL of distilled water colored with 1% basic fuchsine was added. Anthers were crushed and the suspension was shaken to achieve the highest homogeneity. Then we deposited 10 microliters of the suspension in a slide and counted all pollen grains at 400× magnification with an optical microscope. We repeated the pollen count three times for each dilution, considering the average value of the three replicates for each sample. To obtain the number of anthers per flower, we sampled three flowers of three catkins from each tree. To estimate the number of flowers per catkin we counted the total number of flowers in ten catkins of each tree in the studied populations. To estimate pollen production per tree we assessed the number of catkins on three branches of each tree, considering the average of catkins per branch and the number of branches per tree.

2.3. Airborne Pollen Study

Airborne pollen has been continuously monitored over a period of 27 years (from 1993 to 2019) by means of a 7-day VPPS 2000 volumetric pollen trap (LANZONI, Bologna, Italy) [34], located on the roof of the Sciences Faculty in Ourense (approximately 20 m above ground level). The device has a vane tail (orientating the sampler according to the wind direction) and a built-in vacuum pump that has a suction flow rate of 10 L/min. By passing through a narrow intake orifice (2 × 14 mm), the sampled air impacts a clock-driven drum rotating at an angular velocity of 2 mm/h, taking 7 days to perform a complete turn. The drum is covered with a Melinex tape coated with an adhesive solution where airborne pollen is retained. After exposure, this tape is cut into segments 48 mm long, representing 1 day of sampling, that are then mounted on a microscopic slide with a glycerogelatine-basic fuchsin mounting media and a cover glass. Pollen counts were conducted following the methodology proposed by the Spanish Aerobiological Network (REA) [35] based on four equidistant longitudinal transects along the slides. Pollen data were expressed as pollen grains per cubic meter of air (pollen grains/m^3). The Main Pollen Season (MPS) was determined using the Andersen [36] method, which defines MPS as the period from the accumulated sum of daily mean pollen concentrations that reach 2.5% of the annual total to the day when 97.5% of the annual total is achieved. The main characteristics of the airborne oak MPS were estimated during the studied period: start date, end date, length in days, annual pollen integral, and the annual pollen peak and its date.

2.4. Meteorological Data and Statistical Analysis

Meteorological data were obtained from the Galician Institute for Meteorology and Oceanography METEOGALICIA "Ourense" station, placed at 300 m of the pollen sampler. Measured parameters were temperature (°C), relative humidity (%), rainfall (mm) and sun hours (h). With the aim to ascertain the degree of association between the studied reproductive variables of the different sampled oak species, a non-parametric Spearman's correlation test was carried out (considering p values of $p < 0.1$ and $p < 0.05$). The analysis was conducted considering the single species and all species as a whole with the objective to detect possible reproductive strategies. In addition, a linear regression analysis was conducted to estimate increase or decrease trends of the aerobiological and meteorological variables during three different periods (1993–2019; 1993–2004 and 2005–2019). Statistical analysis was performed by means the STATISTICA 7 10.0 program (Dell, Newton, MA-Boston, USA).

3. Results

3.1. Pollen Production Study

The pollen production study of the six oak species analyzed in different forests around the city of Ourense showed wide variations between species (Table 1 and Figure 2).

Table 1. Average, minimum (Min), and maximum (Max) values, standard deviation (S.D.) and the relative standard variation in percentage (%RSD) of the pollen per anther, anthers per flower, flowers per catkin, pollen per catkin, catkins per tree and pollen per tree of the studied *Quercus* species (*robur, pyrenaica, rubra, faginea, ilex* and *suber*) in the North-western Spain.

	Pollen/Anther	Anther/Flower	Flowers/Catkin	Pollen/Catkin	Catkins/Tree	Pollen/Tree $\times 10^9$
Q. robur	**3400**	**8**	**21**	**569,803**	**667,231**	**435**
Min	2093	7	17	394,396	29,250	16
Max	4192	9	25	777,555	2,808,000	1937
SD	651	1	2	132,992	995,721	679
RSD%	19.1	9.6	11.7	23.3	149.2	155.9
Q. pyrenaica	**3457**	**8**	**23**	**623,494**	**249,716**	**142**
Min	2280	8	17	451,656	13,905	12
Max	4596	8	26	897,539	752,123	339
SD	847	0	3	159,916	241,522	127
RSD%	24.5	0.0	12.4	25.6	96.7	89.4
Q. rubra	**4515**	**5**	**31**	**716,385**	**5021**	**3**
Min	2707	5	25	339,036	2160	1
Max	6333	5	36	1,144,272	7700	6
SD	1169	0	4	264,479	2195	1
RSD%	25.9	0.0	13.1	36.9	43.7	49.5
Q. faginea	**4891**	**7**	**15**	**511,301**	**4128**	**2**
Min	2943	6	12	279,173	2160	0.603
Max	5703	8	17	749,139	7700	3
SD	1304	1	3	201,767	2448	1
RSD%	26.7	15.5	17.5	39.5	59.3	54.1
Q. ilex	**6282**	**6**	**13**	**512,987**	**3770**	**1**
Min	3280	6	9	215,253	288	0.175
Max	11,733	6	16	1,020,513	20,400	5
SD	2414	0	2	216,750	4914	1
RSD%	38.4	0.0	14.1	42.3	130.3	100.2
Q. suber	**4536**	**5**	**16**	**355,237**	**16,879**	**7**
Min	3454	4	12	234,885	160	0.040
Max	5521	6	19	625,753	69,433	30
SD	847	1	3	141,804	26,104	12
RSD%	18.7	13.0	17.9	39.9	154.6	154.2

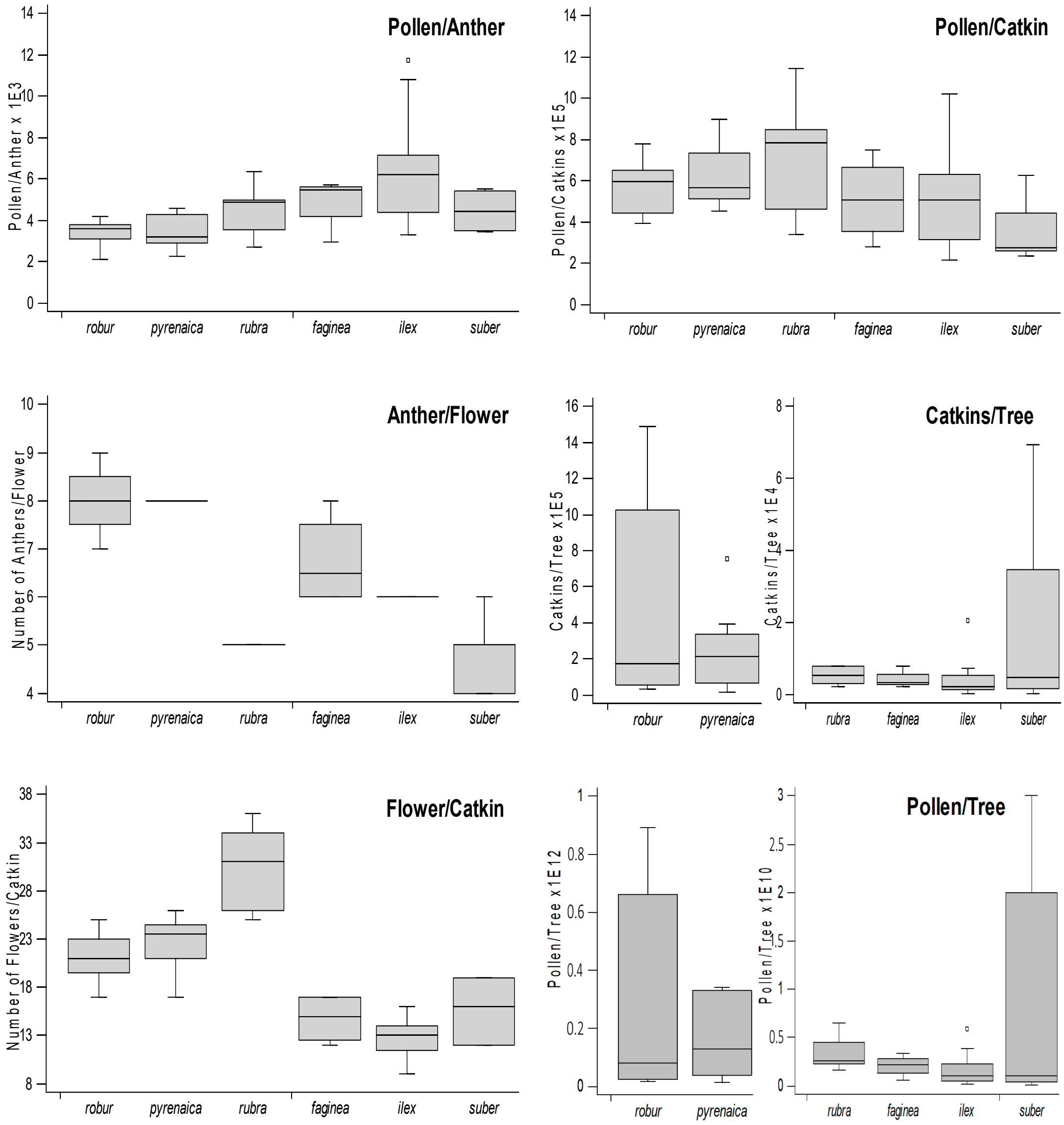

Figure 2. Box-plots of pollen per anther, anthers per flower, flowers per catkin, pollen per catkin, catkins per tree and pollen per tree of the studied *Quercus* species (*robur, pyrenaica, rubra, faginea, ilex* and *suber*). Median (black line), Box: 25–75%, Non-outlier rang (Whisker) and outliers (point). Catkins per tree and pollen per tree plots of *Q. robur* and *pyrenaica* values were showed separately in order a better visibility of the scale.

The average pollen amount per anther ranged between 3400 pollen grains registered for *Q. robur* to 6282 recorded in *Q. ilex*. The lowest relative standard deviation between trees of the same species was estimated for *Q. suber* and the highest for *Q. ilex*. The number of anthers per flower ranged between 5 and 8, with the highest quantity registered for *Q. robur* and *Q. pyrenaica* and the lowest for *Q. rubra* and *Q. suber*. The standard deviations between the sampled trees of a given species were the lowest among the six analyzed flowering parameters. Regarding the quantity of flowers per catkin the highest number was estimated for *Q. rubra*, whilst the lowest was estimated for *Q. ilex*. The number of pollen grains produced per catkin fluctuated from 355,237 pollen grains obtained for *Q. suber* to 716,385 pollen grains registered for *Q. rubra*.

Table 2. Spearman's correlation coefficients between pollen per anther, anthers per flower, flowers per catkin, pollen per catkin, catkins per tree and pollen per tree considering the oak species and the data altogether. Not significant correlations were observed for *Q. faginea*.

	Pollen/Anther		**Anther/Flower**		**Flowers/Catkins**		**Pollen/Catkin**		**Catkins/Tree**	
	Spearman	***p Value***	***Spearman***	***p Value***	***Spearman***	***p Value***	***Spearman***	***p Value***	***Spearman***	***p Value***
Anther/Flower	−0.250	0.080								
Flowers/Catkins	−0.315	0.026								
Pollen/Catkin	0.493	0.000			0.540	0.000				
Catkins/Tree			0.502	0.000						
Pollen/Tree			0.491	0.000					0.994	0.000
Q. robur										
Catkins/Tree			0.772	0.041						
Pollen/Tree									0.976	0.010
Q. pyrenaica										
Pollen/Catkin	0.738	0.051								
Q. rubra										
Flowers/Catkins	0.815	0.046								
Pollen/Catkin	0.857	0.036			0.964	0.018				
Q. ilex										
Pollen/Catkin	0.900	0.001			0.576	0.026				
Pollen/Tree									0.900	0.001
Q. suber										
Pollen/Catkin			0.810	0.047						
Catkins/Tree							0.857	0.036		
Pollen/Tree							0.857	0.036		

The relative standard deviation between trees of the same species ranged from 23.3% to 42.3%. The number of catkins per tree showed important variations as a consequence of the size of mature individual trees in each oak specie. The highest catkin production was estimated for *Q. robur* with 667,231 catkins, whereas the lowest was detected for *Q. ilex* with only 3770. The number of catkins per tree was the parameter with the largest variations of the relative standard deviation. Finally, the highest number of pollen grains per tree was estimated for *Q. robur* with an average of 436 billion pollen grains per tree and the lowest was observed for *Q. ilex* with only 1.6 billion pollen grains produced. Important differences regarding the relative standard deviation were also registered in the case of pollen produced per tree.

Spearman's correlation analysis was applied to ascertain the degree of association between the evaluated flowering parameters, considering data of the six oak species altogether, and several significant correlations were found (Table 2). Negative, significant correlations were observed between pollen grains per anther with number of anthers per flower as well as number of flowers per catkin. A positive, significant correlation was obtained between pollen grains per anther and pollen grains per catkin. The number of anthers formed per flower positively correlated with the number of catkins per tree and pollen production per tree. The number of flowers per catkin positively correlated with pollen grains per catkin. Finally, a positive correlation was found between the number of catkins per tree and the final pollen production per tree.

3.2. Airborne Pollen Study

Oak airborne pollen was present in the atmosphere of North-western Spain during spring, mainly in March, April and May (Figure 3). The average seasonal behavior of oak pollen in the atmosphere is shown in Figure 2. During the 27 years of study, the MPS started on March 26th and ended on June 7th (Table 3).

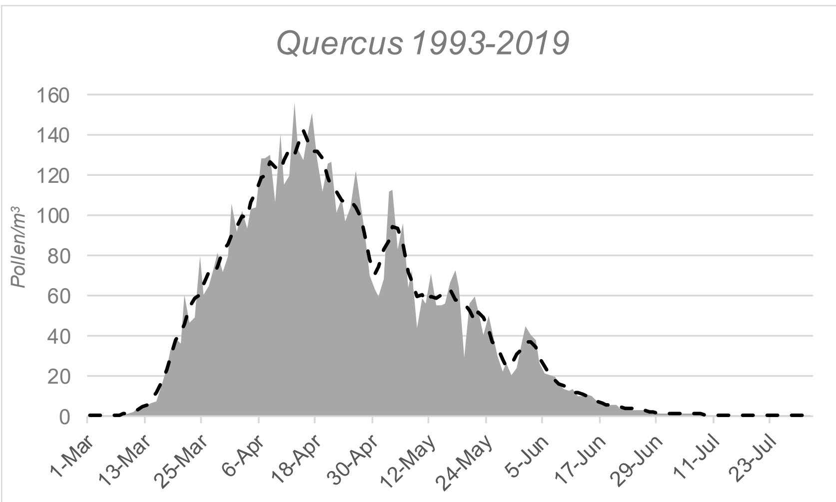

Figure 3. Average daily values of airborne *Quercus* pollen concentration in the atmosphere of Ourense from 1993 to 2019 (in line represent the 5 days running mean).

A similar standard deviation, around 11%, was detected for both dates. The earliest onset was observed on March 11th 1997 and the latest on April 19th 2018. The earliest end date was detected on May 20th 1997, and the most delayed was at the end of June in 2013. Therefore, the oak flowering season covers a long period, with an average duration of 75 days in the studied years, fluctuating from 53 days registered in 2011 and 100 days recorded in 2016 (Table 3).

Table 3. Characteristics of the airborne oak MPS during the 27 years of study: start date, end date, length, annual pollen integral (pollen grains), daily pollen peak (pollen/m^3) and its date. Average value (Mean), minimum value (Min), maximum value (Max), standard deviation (SD) and relative standard variation in percentage (%RSD) of sampled trees for each species were also shown.

	Date Start MPS	Date End MPS	Length MPS	Annual Pollen	Pollen Peak	Date Pollen Peak
1993	18-Mar	16-Jun	91	1625	135	19-Apr
1994	19-Mar	10-Jun	84	1468	115	30-Mar
1995	25-Mar	22-May	59	3180	194	14-Apr
1996	30-Mar	6-Jun	69	2266	215	16-Apr
1997	11-Mar	20-May	71	2816	151	6-Apr
1998	14-Mar	17-Jun	96	1296	77	21-Mar
1999	31-Mar	15-Jun	77	1396	138	5-Apr
2000	14-Mar	15-Jun	94	2392	154	7-Apr
2001	21-Mar	9-Jun	81	1323	104	16-Apr
2002	26-Mar	13-Jun	80	4824	361	1-Apr
2003	17-Mar	27-May	72	2598	218	7-Apr
2004	21-Mar	10-Jun	82	2617	147	4-Apr
2005	1-Apr	7-Jun	68	2716	220	7-Apr
2006	7-Apr	7-Jun	62	5271	330	26-Apr
2007	4-Apr	6-Jun	64	5862	407	19-Apr
2008	27-Mar	18-Jun	84	5772	402	6-Apr
2009	18-Mar	2-Jun	77	13982	1212	21-Mar
2010	12-Apr	6-Jun	56	4924	410	28-Apr
2011	1-Apr	23-May	53	11675	703	6-Apr
2012	24-Mar	7-Jun	76	10138	1017	30-Mar
2013	7-Apr	30-Jun	85	9649	568	24-Apr
2014	30-Mar	30-May	62	15115	1250	16-Apr
2015	2-Apr	10-Jun	70	9290	642	13-Apr
2016	16-Mar	23-Jun	100	7560	541	3-May
2017	18-Mar	25-May	69	18269	1061	10-Apr
2018	19-Apr	19-Jun	62	11343	866	6-May
2019	21-Mar	8-Jun	83	9085	567	31-Mar
Mean	***26-Mar***	***7-Jun***	***75***	***6239***	***452***	***11-Apr***
Max	***19-Apr***	***30-Jun***	***100***	***18269***	***1250***	***6-May***
Min	***11-Mar***	***20-May***	***53***	***1296***	***77***	***21-Mar***
SD	***10.90***	***10.87***	***13.22***	***5182.23***	***379.67***	***13.09***
RSD%	***0.03***	***0.02***	***17.61***	***83.06***	***83.99***	***0.03***

Oak pollen represented, on average, around 14% of the annual total pollen registered in the atmosphere. The percentage varied substantially from year to year, but the highest rates were observed during the last decade (Figure 4).

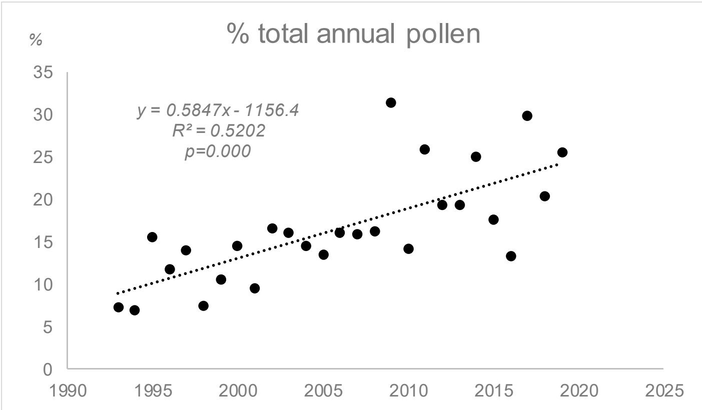

Figure 4. Trend of the percentage of airborne *Quercus* pollen concentrations from 1993 to 2019 regarding to the total pollen content in the atmosphere.

The annual pollen integral was also highly variable, ranging between 18,269 pollen grains registered in 2017 to 1296 observed in 1998 (Table 3). Peak pollen concentration was generally observed in April, with the latest record on 16 April 2014, with 1250 pollen/m^3.

The intensity and temporal trends of the oak MPS features and the main meteorological parameters considering the entire year and the three months prior to flowering (January, February, and March), were calculated by means of simple linear regressions (Table 4).

Table 4. Trends of the MPS for pollen (start, end and length dates of the MPS, annual pollen integral, pollen peak concentration and pollen peak day) and for meteorological variables (Maximum, Mean and Minimum temperatures, Total rainfall, Relative Humidity and Sun hours) during the whole data set and during the 1993–2004 and 2005–2019 periods. The shown parameters of the linear regression analysis were slope, R^2 value and p-value (in bold $p < 0.01$, $p < 0.05$).

Pollen	1993–2019			1993–2004			2005–2019		
	Slope	R^2	p	*Slope*	R^2	p	*Slope*	R^2	p
Start MPS	**0.471**	**0.149**	**0.046**	0.011	0.001	0.985	0.389	0.031	0.529
End MPS	0.139	0.012	0.581	0.185	0.004	0.832	0.311	0.018	0.635
Length MPS	−0.306	0.038	0.328	0.178	0.003	0.854	0.775	0.074	0.326
Annual Pollen	**490.37**	**0.661**	**0.000**	93.367	0.108	0.297	**551.721**	**0.337**	**0.023**
Pollen peak	**34.37**	**0.583**	**0.000**	6.112	0.088	0.349	33.235	0.205	0.089
Peak day	0.3492	0.0509	0.257	−0.581	0.065	0.429	0.239	0.005	0.791
Year	*Slope*	R^2	p	*Slope*	R^2	p	*Slope*	R^2	p
Tmax	0.029	0.108	0.093	0.046	0.078	0.378	0.022	0.017	0.642
Tmed	−0.008	0.018	0.506	0.034	0.042	0.519	0.033	0.102	0.244
Tmin	0.0129	0.0301	0.385	0.041	0.074	0.393	0.034	0.067	0.348
Rainfall	−7.626	0.094	0.127	−17.609	0.105	0.304	10.396	0.061	0.374
Humidity	−0.0978	0.056	0.194	−0.304	0.073	0.394	0.079	0.037	0.489
SunHours	0.0247	0.124	0.071	−0.097	0.306	0.062	0.018	0.057	0.388
Jan-Feb-Mar	*Slope*	R^2	p	*Slope*	R^2	p	*Slope*	R^2	p
Tmax-JFM	**−0.058**	**0.1627**	**0.037**	0.015	0.003	0.863	−0.037	0.022	0.596
Tmed-JFM	**−0.071**	**0.319**	**0.002**	0.038	0.027	0.606	−0.096	0.249	0.057
Tmin-JFM	−0.014	0.008	0.651	0.057	0.027	0.608	0.003	0.001	0.963
Rainfall-JFM	3.748	0.047	0.278	7.727	0.031	0.581	9.666	0.121	0.204
Humidity-JFM	−0.012	0.001	0.712	−0.112	0.005	0.823	0.163	0.044	0.451
Sun Hours-JFM	−0.021	0.032	0.375	−0.089	0.155	0.231	−0.021	0.008	0.751

With the purpose to achieve a deeper understanding of the climate change effects on oak flowering, trends were calculated using the entire 27 years of data (1993–2019) as well as considering separately the first 13 years (1993–2004) and the last 15 years (2005–2019) of the study period. Considering the entire study period, some significant statistical trends were obtained in the exploratory analysis, showing a delay in MPS onset of 0.47 days per year, and an increase in the annual pollen integral of the 7.9% of pollen grains per year (Figure 5).

The total rainfall recorded during January, February and March seemed to strongly affect the total oak pollen integral (Figure 6). Regarding the two periods considered separately, significant trends were registered only for the annual pollen integral and the pollen peak during the last 15 years. No statistically significant trends were observed for the exploratory analysis of meteorological variables over the years, with the exception of the increase in maximum temperatures in the entire data set (Table 4). Considering meteorological data of the three previous months to oak flowering, significant trends of maximum temperature decrease (−0.058 °C per year) and mean temperature decrease (−0.071 °C per year) were observed.

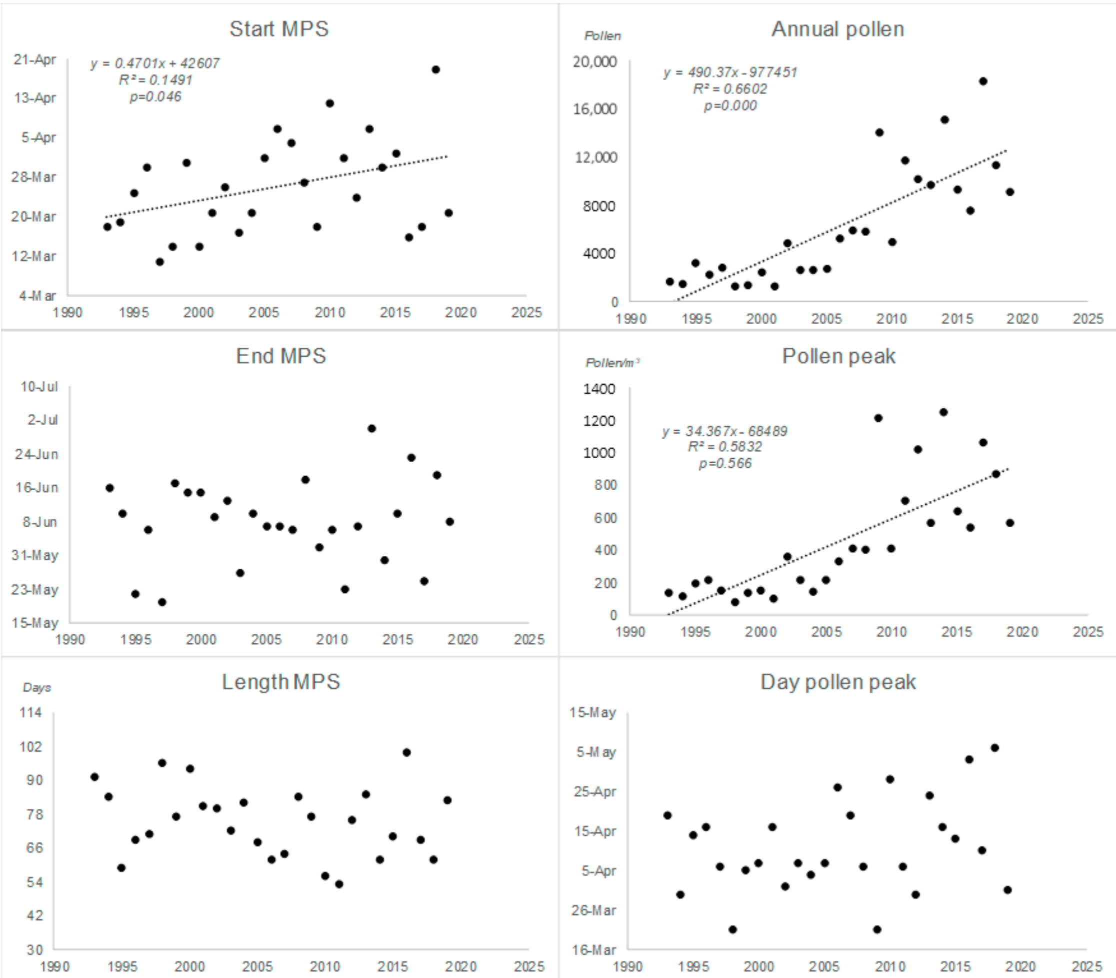

Figure 5. Trends of the airborne *Quercus* Main Pollen Season (MPS) characteristics during the 27 years of the study: start date, end date, length, annual pollen integral, daily pollen peak and its date.

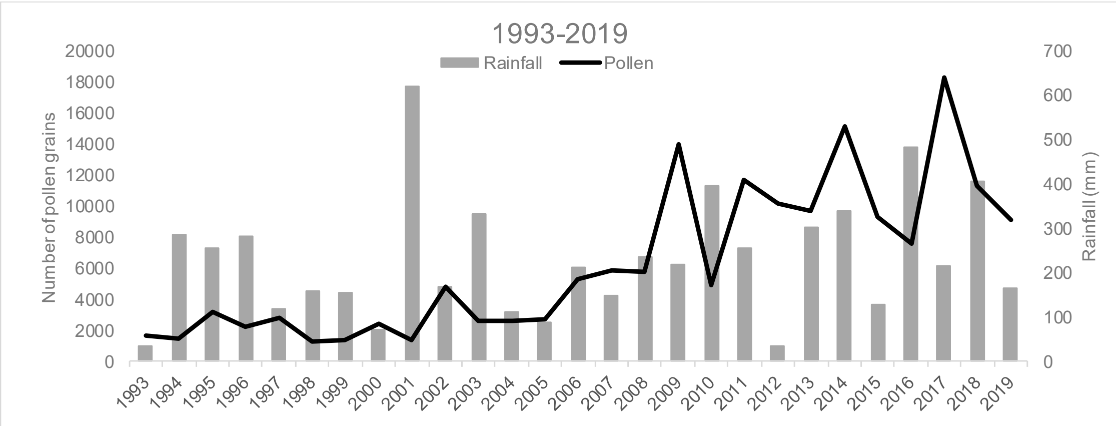

Figure 6. Relationship between accumulated rainfall during the months of January, February and March with the annual *Quercus* pollen integral in the atmosphere.

4. Discussion

The main characteristics of wind as a pollination vector are its lack of specificity and its random dispersal mechanism. Therefore, anemophilous trees stimulated the production of high pollen amounts to facilitate fertilization processes as a response to the wind-limited efficiency. Successful flowering depends not only on abiotic parameters, such as meteorological factors, and tree biotic features that are genetically regulated [37] such as the number of pollen grains per anther, anthers per flower, flowers per catkin, and catkins per tree [3,38].

Our study showed the amount of pollen produced by individual members of six oak species, which is approximately summarized in the proportions 1:1:2:5:90:276 for *Q. ilex*, *Q. faginea*, *Q. rubra*, *Q. suber*, *Q. pyrenaica* and *Q. robur* respectively. Previous studies have rarely focused on pollen production in the two more prominent species (*Q. robur* and *Q. pyrenaica*) represented in oak forests of North-western Spain; they only examined their pollen viability characteristics [39]. Some research has been conducted in order to measure the pollen production of *Q. ilex* [11,24], *Q. suber* and *Q. faginea* [11] in the Mediterranean basin. In the case of pollen per anther and pollen per flower production, previous studies verified lower values of pollen production than that registered in our research for *Q. ilex*, *Q. suber* and *Q. faginea* [11,24]. The amount of pollen produced per catkin or per tree was similar to that obtained in North-western Spain for *Q. ilex,* whilst it was higher in the cases of *Q. suber* and *Q. faginea* [11,24]. The number of anthers formed per flower or catkin registered in the study area, at the limit of the *Q. ilex*, *Q. suber* and *Q. faginea* distribution, was lower than that observed for trees of the same species located in their optimal Mediterranean bioclimatic area [11,24]. These high values were similar to the observed for the North Spain oak species, *Q. robur* and *Q. pyrenaica*, located in their optimal bioclimatic conditions [11,24].

Several statistically significant associations have been registered between the evaluated flowering parameters, with higher correlation coefficients when data of all species were considered altogether. Our study showed that higher pollen production per anther is associated with a lower quantity of anthers per flower and flowers per catkin, which is in accordance with the results pointed out by Tormo [24]. The reproductive strategy of the *Quercus* genus consists of a higher production of pollen per anther in tree species with smaller size, mainly in the case of perennial species, in order to ensure fertilization. It was pointed out that pollen production per anther appears to be genetically fixed [37]. Another detected pollination approach was that a higher number of anthers per flower coincided with a higher number of pollen grains per tree. In our study, the number of anthers per flower was higher in deciduous species, such as *Q. robur*, *Q. pyrenaica* and *Q. rubra,* which presented the highest number of flowers per catkin. On the contrary, studies conducted by Tormo [24] noted that a higher number of anthers or flowers per catkin, corresponded to the production of a lower number of catkins by trees. In our study, statistical correlation revealed that species with lower pollen per anther production presented a higher number of flowers on each catkin, and species with a higher production of pollen per catkin presented a higher number of flowers in the catkin. Our data also showed a highly positive correlation between the number of catkins per tree and total production of pollen per tree. Studies conducted by Tormo [24] noted the same behavior, as teller trees increase their production of anthers, flowers, catkins and pollen. Oak species showed a tendency to compensate their sexual characteristics (such as pollen grains per anther, or inflorescences per tree) by increasing some of them or reducing others [11]. Nonetheless, in the study area *Q. robur* and *Q. pyrenaica* were the species with the largest pollen production as a consequence of their height and tree-crown diameter. In fact, pollen production depends on the production rate of pollen grains for individual plants, which in turn depends on the genotype, age, plant size, phenology, regional climatic and edaphic factors, diseases, and so forth [12,40–42].

Information about the total pollen production per oak species, supported by knowledge on the coverage of various species in a given area, is useful to estimate the number of pollen grains in the atmosphere [11,24,43]. The curve of daily mean values of airborne oak pollen in the study area was characterized by several concentration peaks corresponding to successive flowerings of different species [2,44]. *Quercus robur* was the first species to flower in Northwest Spain; therefore, onset of the pollen season refers to this species. *Q. robur* pollination was closely followed by *Q. faginea*, *Q. pyrenaica* and *Q. ilex* flowerings [44]. The last species to flower was *Q. suber*, whose pollination period did not overlap with any flowering period of other oak species. Therefore, the pollination period is very long as a result of the abundance of late-flowering oak species such as *Q. ilex* and *Q. suber* [7].

Regarding annual totals, oak pollen in the atmosphere of the study area has shown an increasing trend during recent years. This tendency could denote the restoration of oak natural forests as a sign of ecological maturity in the overall vegetation distribution [2,4,44]. Previous studies in the study area pointed out a continuous growing trend of autochthonous oak forests during the end the 20th century and in the first decade of the 21st century mainly resulting from changes in land use through reforestation or from natural regeneration of abandoned agricultural land [31]. Other factors, such as, forest fires or agricultural practices, could also have caused for this change [42,45,46]. In contrast, oak natural forests have been almost completely replaced by non-irrigated olive groves, reducing their representation in the atmosphere in some Mediterranean areas [12].

Pollen rains from anemophilous plants induce rapid pulses of nutrients into the food web of ecosystems [16]. Several decomposers, such as fungi, bacteria, protozoans, and various groups of invertebrates, use pollen pulses as an easily available, digestible, and nutritious food, facilitating the entry of limiting nutrients within and across ecosystem nutrient cycling [16]. Forests may produce pollen in masses reaching 100 to 1000 kg/ha [13] increasing the productivity of the ecosystem by the addition of non-C limiting elements, such as P, K, S or Mg, even at a distance of thousands of kilometres between ecosystems [47]. Thus, estimation of the pollen mass from the main wind-related pollination forest in the study area should be included when calculating the annual biomass input in ecosystems. Scarce studies in scientific literature have considered the production of oak pollen in mass units, pointing out the mean mass of a single oak pollen grain as 0.011 µg as average [48]. This value allows us to calculate the mass of pollen produced in our area of study where the most prominent forests belongs to *Q. robur*, *Q. pyrenaica* and *Q. ilex* species. Considering the registered pollen production per tree, the natural forested cover of the three species [31] (as the other oak species occupied low surfaces due to their ornamental use), and the number of tress per ha (ranging from 516 for *Q. robur* to 696 for *Q. pyrenaica* [31]), the mass of pollen produced by the forests was estimated. An amount of 157×10^6 kg/ha was produced by *Q. robur*, 121×10^6 kg/ha in the case of *Q. pyrenaica* and only 0.00009×10^6 kg/ha for *Q. ilex* pollen mass introduced in the ecosystem food webs. This information would be valuable in the contexts of nutrient cycling and food web functions in forests. In addition, pollen from wind-pollinated trees, including various oak species, is a very important source of "high-quality" larval food for both solitary wild bees and managed honey bees [49]. Bees and larvae may be limited by the availability of P, Na, Mn, Mg, K, Fe, Ca, Zn and Cu [50], which are essential nutrients present in airborne pollen grains.

The large mass of pollen produced by oak forests in the study area could help satisfy the demand for a nutritionally balanced diet in growing bees [51].

Increases in oak pollen concentrations in the atmosphere could also have a negative impact, as a consequence of the related development of sensitization in allergic people. Some researchers considered this pollen type responsible for major cases of allergies in areas with abundant oak vegetation [21,25]. The maximum concentration values were generally attained between the last week of March and the first two weeks of April, coinciding with the flowering of the most abundant species in the area, *Q. robur* and *Q. pyrenaica* [44]. Both species were the main contributors to the oak pollen curve, although *Q. ilex* was responsible for 10% of airborne pollen in years with high pollen production and favorable weather conditions [3]. Peak concentrations were produced slightly later in certain Mediterranean areas as a consequence of cork oak abundance [7].

Due to the high representation of oak trees in Northwestern Spain, the annual pollen integral was very high. Previous studies detected statistically significant differences between the oak annual pollen integral in the Eurosiberian region and those of the rest of the Iberian Peninsula [7]. Similar values were detected in the Northeast [52], while higher values were noted in the central part, where up to 66,000 pollen grains were registered in some years [53], and lower values were noted in the South [4,54]. Pollen production and dispersion is strongly modulated by a complex combination of biotic and environmental factors [12]. Annual pollen integral differences between years could be explained by abiotic factors, such as meteorological conditions, or biotic factors such as biennial behavior rhythms due to an alternation in the mobilization of nutrient reserves towards the tree's vegetative growth or towards the reproductive structures [1,45,55]. The obtained pollen data from 2011–2013, 2014–2016 and 2017–2019 could suggest this behavior, represented by a three-year cycle with one year of high pollen concentration followed by two years with lower values. Fluctuations in pollen production may account for biannual, triannual, or four-year cycles, a phenomenon that has been widely reported for oak pollen [4,56]. Moreover, the influence of other biotic factors may be very important, particularly during rainy years, since the insect *Neuropterus* triggers the development of gall on catkins [57]. A generation of asexual *Neuropterus* emerges from these galls and will deposit spherical galls in young leaves and catkins. In a short time, a new sexual generation with males and females will emerge and form lenticular galls [44]. In our study, years with the lowest pollen values mainly coincided with high accumulation of rainfall during the previous months to flowering. This could also hinder accurate pollination predictions, as under this condition the catkins are moistened, which impedes the formation of pollen grains. This has a higher effect in early flowering oak trees, mainly *Q. robur*, than in late flowering species such as *Q. pyrenaica* [44]. In the rainiest years, galls of the insect can cause half the catkins to develop compared to those that would be formed in a normal year.

During recent years, temperature trends have displayed regional differences with a non-uniform influence on ecological processes [10,58,59]. These trends have much more influence in winter and early spring flowering trees, since in these seasons the temperature registered during the last years has varied widely [4,60]. Our study detected a statistically, significant increasing trend in yearly maximum temperature, whereas a decrease in the maximum and mean temperatures during the three previous months to oak flowering was observed. The main reported changes related to the flowering phenology of plants have been the earlier onset [59,61], a longer pollen season [58,62], and an increase in airborne pollen concentrations [21,63,64]. Previous studies have been conducted in different Spanish localities to ascertain the atmospheric oak MPS behavior and to predict possible variations induced by climate change [4,7,12,18]. Our study showed a statistically significant in delay of MPS onset (0.47 days per year), possibly as a consequence of the decrease in maximum and mean temperatures during the three months preceding oak flowering, mainly during the 2005–2019 period. On the contrary, an earlier onset in some trees, including oaks, has been reported in different European areas [65], including Spain, associated with increased temperatures in the pre-flowering periods [1].

In the present study, a statistically, significant increasing trend was also observed for the annual pollen integral (a 7.9% of pollen grains per year) and for the peak pollen concentration (a 7.5% of pollen grains per year). These changes were also mainly produced during the 2005–2019 period. Increases in oak pollen counts have been observed for many Spanish cities, mainly in inland locations [1]. Some authors argued that the increase in atmospheric CO_2 concentration could stimulate pollen production by plants [66,67]. As a consequence of the rapid response to environmental changes, some authors have proposed oak pollen levels as the best bio-indicators to detect ecological variations induced by climate change [11,12].

Finally, it is important to highlight that oak flowering out of the pollination period was often noticed during the last ten years in North-western Spain. Evidences was found through field phenological observations and the detection of elevated amounts of oak pollen grains in the atmosphere during autumn months. The same behavior was noted in South Spain, where populations of *Q. rotundifolia* and *Q. suber* bloom twice a year [4].

5. Conclusions

Pollen produced by the six studied oak species follow the proportions 1:1:2:5:90:276 for *Q. ilex*, *Q. faginea*, *Q. rubra*, *Q. suber*, *Q. pyrenaica* and *Q. robur* respectively. Among the evaluated flowering parameters, the number of anthers per flower showed the lowest variation between trees from each oak species, what could reflect the genetic regulation that drives this parameter.

Pollen per anther was negatively correlated with the number of anthers per flower and the number of flowers per catkin. This fact shows the reproductive strategy of oak, since a higher production of pollen per anther compensates the lower number of anthers and flowers formed in order to ensure appropriate fertilization. The number of anthers per flower or catkin per tree registered in the bioclimatic transition region of the study area, at the limit of the *Q. ilex*, *Q. suber* and *Q. faginea* distribution, reflected a condition of boundary closeness, with a lower anther formation than that noted for trees of the same species located in their optimal Mediterranean bioclimatic area. This was also observed for *Q. robur* and *Q. pyrenaica*, which presented higher anther formation rates when located in their optimal bioclimatic conditions.

Oak pollen represented around 14% of the total annual airborne pollen, although it varied substantially from year to year, and recorded an increasing trend during the last decade. This could denote the restoration of oak natural forests as signal of ecological maturity in vegetation distribution, but it can have a negative impact on oak pollen-related allergies, since this genus is considered responsible for major cases of allergies in areas with abundant oak vegetation.

Additionally, the possible use of oak pollen levels as a bio-indicator to detect ecological variations induced by climate change is remarkable due to its rapid response to environmental stimulus. Considering the 1993–2019 data set, we observed a significant delay in the MPS onset of 0.47 days per year, which could be related to the decreasing trend observed in maximum and mean temperature during the three previous months to flowering (January, February, and March). Moreover, we also found a significant increasing trend of a 7.9% of pollen grains per year in the annual pollen integral, and a 7.5% in the case of the peak pollen concentration per year, which was noticeable during the last fifteen years in the study period (2005–2019 data set).

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