

## Article

# Reproductive Biology of Olive Trees (*Arbequina* cultivar) at the Northern Limit of Their Distribution Areas

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**Abstract:** In recent years, North-western Spain has experienced an increase in the cultivated area of olive trees. The main propitious areas for olive groves are the Miño and Sil basins, as a consequence of their Oceanic climate with Mediterranean influence. The objective of this study is to determine the characteristics of reproductive biology, phenological and aerobiological behaviour of olive trees in the most northerly new plantation areas of the Iberian Peninsula. The study was carried out in an olive grove growing *Olea europaea* L. cv. ‘Arbequina’ located in Quiroga (Lugo) from 2016 to 2018. The phenological observations were based upon the main growth stages following the Biologische Bundesanstalt Bundessortenamt and Chemical industry (BBCH) scale. To predict the onset of flowering, a thermal time model was used in order to quantify the chill requirements, and growing degree-days were applied to determine the heat requirement. The production, viability and germination rates of *Olea* pollen were evaluated from samples selected in nine individual trees for the phenological survey. The aerobiological study was conducted by means of a Hirst-type pollen trap located in the centre of the olive grove. The vegetative period of the olive tree in the study area lasted an average of 259 days. The important phenological stage 6 (flowering) was the shortest stage. An average of 704 Chilling Hours (CH) with a threshold of 2.5 °C was required to overcome the chilling period, 1139 Growing Degree Days (GDD) for the beginning of flowering, and 4463 GDD for harvest. The pollen production per anther was 82589 grains ( $\pm$  14084 pollen grains), with a rate of 81% viability and 12% pollen tube germination. The main pollen season started on average on May 20th and ended on June 16th with an average duration of 27 days and an annual pollen integral of 833 pollen grains. The low pollen concentrations could be a consequence of the Northern location of the forest, in a bioclimatic transition zone between the Eurosiberian and the Mediterranean areas, at the limit of olive tree distribution.

**Keywords:** aerobiology; dormancy; olive; phenology; pollen; viability; germination; production



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## 1. Introduction

The olive tree (*Olea europaea* L.) is one of the most widespread tree species in the Mediterranean basin, exerting a great economic and social influence through its fruit and its oil [1]. Spain is the largest producer of olive oil with 42% of the total world production with the main cultivation areas located in the centre and south of the Iberian Peninsula [2]. In recent years, North-western Spain has experienced an increase in the cultivated area of olive trees, for both ornamental and industrial uses, contributing to rural development as one of the major sources of income and employment in this area [3,4]. The most propitious areas for olive groves are the Miño and Sil basins, as a consequence of their Oceanic climate with Mediterranean influence.

Climatic conditions are the greatest determining factor for olive cultivation [5], mainly temperature, precipitation and relative humidity, variables that directly condition the phenological response of the tree [1,5–7]. Phenological studies allowed us to know the periodical rhythms of biological events and their relationships with the environment [8]. The onset of phenological events in arboreal plants is more closely related to thermal time rather than to calendar time [9,10]. In the case of olive trees, phenology is characterized by the formation of buds in summer, dormancy in autumn, budburst in late winter and flowering in late spring [1,11]. To survive periods of adverse climatic conditions, the growth of woody plants in temperate regions slows down, entering into a physiological state known as “dormancy” [10] in which the cell water is prevented from freezing [12]. The release of floral bud dormancy occurs when olive trees have been exposed to chilling temperatures for a sufficient time depending on the biogeographic area [8,11]. Once this chilling is fulfilled, certain amounts of heat-thermal units are required for the correct development of inflorescence [13]. The main difficulty of the agrometeorological models is to ascertain the starting date of the accumulation period of Growth Degree Days (GDD) for the heat units as a consequence of climatic variations between different geographical areas. The transition between endodormancy and eco-dormancy is not easily distinguishable, although physiological and anatomical changes were identified in olive flower buds during dormancy when compared to vegetative buds [13]. The relationship between both chill and heat requirements has been reported by different authors who consider that chilling temperatures accelerate growth renewal once dormancy is broken; the more chilling units are accumulated, the fewer forcing units are needed later for budburst [14,15].

Anemophilous trees generally produce pollen grains in large quantities per individual flower, as an ecological strategy to ensure fertilization processes [16]. The sterility of the pollen could act as a limiting factor in the production of fruits and olives [17] since the viability of pollen under certain conditions can limit the *Olea* yield, leading to significant economic losses [18]. The development of the pollen grain is sensitive to environmental conditions, both abiotic (such as heat, cold or drought) and biotic factors [19]. Thus, the analysis of pollen production and viability also provides valuable information on upcoming harvests [20] by allowing the development of prediction models several months in advance [21,22]. Numerous studies have examined pollen emission as a function of reproductive phenology status, genetic and environmental factors [23–25]. Furthermore, climate change can impact weather-related variables, affecting both the quantity and quality of pollen produced, and therefore influencing its allergenicity [26]. According to many authors considering pollen production, phenological and aerobiological data are useful tools to analyse the flowering stage in plants [25,27,28]. Some variables such as bio-geographical factors [29] or the variety planted [30] affect pollen production. It should be noted that climatic conditions also affect this aspect, as temperature and water availability also affect pollen production [29,31].

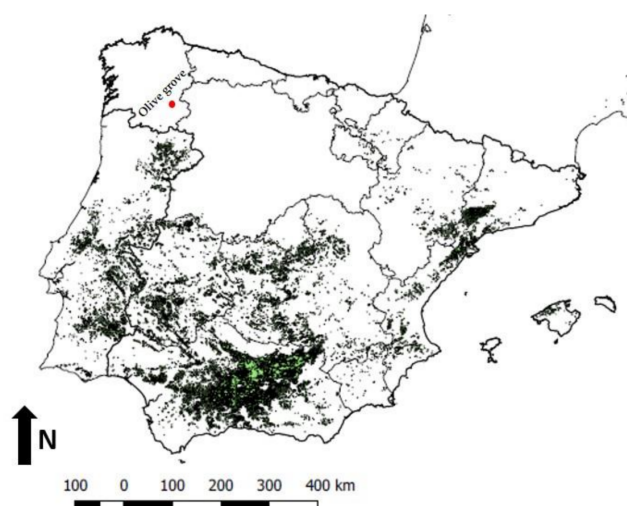
Nowadays, the extension of olive trees in the South of Galicia is 300-ha, distributed at the limit zones of the Mediterranean bioclimatic area. The main cultivar in new plantations is Arbequina [32]. The aim of the study is to determine the characteristics, reproductive biology, phenological and aerobiological behaviour of olive trees in the most northerly new plantations areas of the Iberian Peninsula.

## 2. Materials and Methods

The study was carried out in the olive grove, *Olea europaea* L. cv. ‘Arbequina’, located in Espandariz, Quiroga (Lugo) in the North-western Spain (42°28′33″ N 7°16′18″ O) during the years 2016, 2017 and 2018 (Figure 1). This area is located 267 m above sea level and has a temperate oceanic climate (Cfb) according to the Köppen-Geiger classification [33–36]. This region is influenced by a Mediterranean climate (the minimum amount of rainfall occurs in summer, although not pronounced enough) and with a certain continental influence (Figure S1). The annual average temperature is 11.5 °C and the annual total rainfall is more than 800 mm per year [37]. During the period of study the only variations to this

pattern were registered in 2017 with a lower annual rainfall of 683 mm and annual average temperatures of 14.1 °C.

The phenological observations were based on the main growth stages of the tree [38] following the Biologische Bundesanstalt Bundessortenamt and Chemical industry (BBCH) scale developed by Meier et al. [39] and adapted for olive trees by Sanz-Cortés et al. [40]. For the phenological study, nine trees of the Arbequina variety were selected. The main stages monitored were: Stage 5 (inflorescence development), Stage 6 (flowering), Stage 7 (fruit development) and Stage 8 (fruit maturation). Four principal phenological stages were analysed, and three phenological phases in each stage: stage 5 phases (51: Inflorescence: buds start to swell on stem; 55: Flower cluster expanded, floral buds start to open; 59: The corolla changes from green to white colour); stage 6 phases (61: Beginning of flowering: 10% of flowers open; 65: Full flowering: at least 50% of flowers open; 69: End of flowering, fruit set, non-fertilized ovaries fallen); stage 7 phases (71: Fruit size about 10% of final size; 75: Fruit size about 50% of final size, stone starts to lignificate; 79: Fruit size about 90% of final size, fruit suitable for picking as green olives), and stage 8 phases (81: Beginning of fruit colouring; 85: Increase of specific fruit colouring; 89: Harvest maturity: fruits obtain their typical variety colour, remaining turgid, suitable for oil extraction). A weekly visit to the olive grove was conducted between the months of February and November during the years of the study. For the elaboration of the phenological calendar, the starting date of each stage was considered as when half of the selected trees reached this stage.



**Figure 1.** Location of olive groves in the Iberian Peninsula.

The quantification of chilling and heat requirements to overcome the dormancy period was assessed to predict the start date of the different *Olea* phenological stages. In the present study, to determine chilling requirement, the thermal time model proposed by Aron [41] has been selected:

$$N = 801 + 0.2523B + 7.57B^2 \times 10^{-4} - 6.51B^4 \times 10^{-10} - 11.44\text{minT} - 3.32\text{maxT}$$

N: number of chilling hours;

B: 24D;

D: length of the period in days;

maxT: maximum temperature of the period;

minT: minimum temperature of the period.

In addition, the vegetative activity method (V.A.) was also applied, selecting days in which meteorological conditions did not allow any physiological activity in the tree [42]. The end of chill accumulation was considered when the average temperatures reached their

minimum values and a change in trend was detected by calculating a polynomial line of the second degree between the average temperatures from November to February [43].

The Heat requirements (HR) expressed as Growth Degree Days (GDD, °C) were estimated as a function of the sum of the maximum daily temperatures from the end of the chilling period to the beginning of the different phenological stages, taking into account different threshold temperatures [44].

$$\text{GDD} = \sum \text{maxT} - \text{Th}$$

GDD °C: °C accumulation;  
maxT: maximum daily temperature;  
Th: threshold temperature.

The Ring et al. [45] model was also applied to calculate the heat requirements:

$$\text{GDD} = \sum (\text{avgT} - \text{Th})$$

avgT: daily mean temperature;  
Th: threshold temperature.

The purpose of both methods was to analyse a range of temperatures and the total number of days with a temperature above the threshold temperature (Th) to provide an optimum base temperature for heat accumulation. The lowest standard deviation and coefficient of variation were used to identify the most suitable threshold temperature, both in the chilling and forcing temperature periods.

The production, viability and germination rates of *Olea* pollen were evaluated from samples of two anthers in two flowers close to the anthesis of every nine selected trees. To calculate the number of pollen grains per anther, the Neubauer Chamber methodology was applied [46,47]. The stains used to study the pollen viability were carmine acetic (CA, PanReac) and 2, 3, 5-triphenyltetrazolium chloride (TTC, Merck), identifying the weakly stained cells as non-viable pollen. TTC is a redox indicator especially indicative of cellular respiration that requires previous incubation in a humid chamber and in the dark during 24–48 h at 25 °C. In both cases, the colorimetric reaction was observed under a light microscope (\*10) at least on 100 pollen grains per preparation. The results were expressed as percentages with respect to the ratio between the number of viable pollen grains and the total number of grains [48]. The germination medium (based on Brewbaker and Kwack [49]) contained 20% sucrose, 100 ppm H<sub>3</sub>BO<sub>3</sub>, 300 ppm Ca(NO<sub>3</sub>)<sub>2</sub> and 2% of agar in 1 L of distilled water. For this purpose, plastic Petri dishes with four 90 mm sections (Phoenix Biomedical) were used. The hydrated pollen was distributed in each of the four compartments containing solid medium. To avoid dehydration and enhance surface extension, 10 µL of the liquid culture medium (without agar) was added with a sterile pipette over each quadrant; this also favours the onset of germination [24]. The dishes were incubated in the dark at 25 °C [50]. After 24 h, the plates were directly observed under a Nikon (\*10) light microscope. The in vitro germination rate was determined by counting at least 100 pollen grains per preparation, considering pollen grains with a pollen tube length equal to or greater than the grain diameter as germinated [40,51,52]. After 24 h incubation in the dark at 25 °C, the Petri dishes were observed under a light microscope at x100 magnification.

The aerobiological study was conducted by a Lanzoni VPPS 2010 pollen trap [53] located in the centre of the olive grove during the months of May and June. The methodology proposed by the REA (Spanish Aerobiological Network) was applied, based on four longitudinal transects along the slides [54]; the total values were expressed as pollen grains and daily mean values as pollen/m<sup>3</sup>. The main pollen season (MPS) was considered to be the period during which 95% of the total accumulated annual sum of pollen is recorded. MPS begins when the accumulated sum of pollen reaches 2.5% of the annual total and ends on the day when 97.5% of the annual total is reached [55]. Statistical analysis was

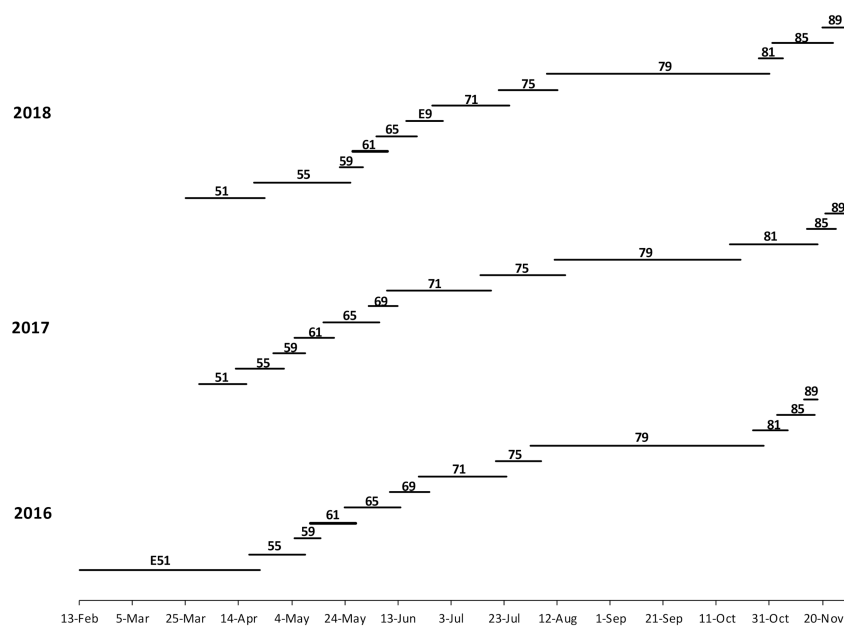
performed using a non-parametric Spearman correlation test in order to assess the correlations between meteorological factors and pollen concentrations. Factor Analysis of Principal Components (PCA) was also carried out in order to detect any structure in the relationships between variables, as well as to categorize the variables according to their influence on the variation of the dependent variables [56,57] and to know the influence of the meteorological variables as a whole. For statistical analysis, the STATISTICA 7.1 package was used.

The meteorological data were obtained from the San Clodio Ribas de Sil MeteoGalicia Station located 1 km from the olive grove. Minimum, average and maximum temperature, relative humidity, dew point, rainfall and wind speed were used for the study.

### 3. Results

The vegetative period of the olive tree in the study area lasted an average of 259 days. The year with the longest cycle was 2016 with 280 days and the shortest was detected in 2017 with 247 days (Supplementary material Table S1).

Stage 5 of inflorescence development occurred an average between mid-March and the second week of May depending on the year of study (Figure 2). The earliest start date was detected in 2016 from mid-February to mid-May, a total duration of 92 days. During the years 2017 and 2018, the onset of stage 5 was detected slightly later, in the last days of March, with a shorter duration of 40 days and 67 days, respectively (Table S1). The important phenological stage 6 of flowering was the shortest. Depending on the year of study, the start of flowering takes place from the second tenth of May to mid-June. This stage had a similar duration during the three years of the study being somewhat longer in 2016 with a length of 41 days, almost one more week. The phenological stage 7 (fruit development) took place from the second half of June to the second half of October, being the longest stage with a duration of between 123 and 29 days (Table S1). Finally, stage 8 fruit ripening, covers the period from mid-end October to when the olive harvest took place, on different dates, 18th November 2016, 2nd December 2017 and 30th November 2018, with a length between of 21 to 43 days (Table S1, Figure 2).



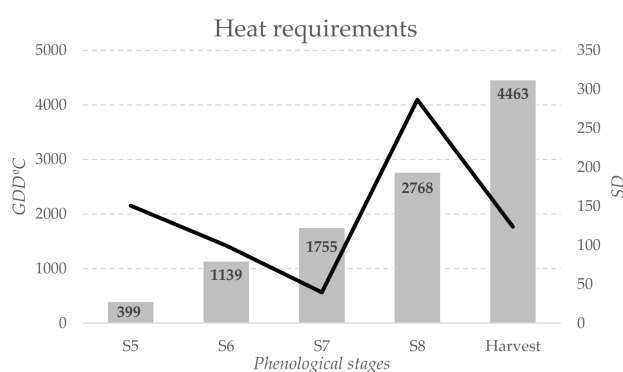
**Figure 2.** Length of the different phenological phases (Stage 5 phases 51, 55, 59; Stage 6 phases 61, 65, 69; Stage 7 phases 71, 75, 79 and stage 8 phases 81, 85, 89) during the three years.

To predict the onset of flowering, a thermal time model was used in order to quantify the chill requirements (Table 1), and growing degree-days was applied to determine the heat requirement.

**Table 1.** Chilling requirements (chilling hours) obtained by means of the vegetative activity (V.A.) method (Jato et al. [42]). Threshold temperature (Th), mean, maximum, minimum, standard deviation (SD) and coefficient of Standard deviation in percentage (C.S.%).

| Chill Requirements |      |      |      |      |     |      |       |       |
|--------------------|------|------|------|------|-----|------|-------|-------|
| Th °C              | 2016 | 2017 | 2018 | Mean | Min | Max  | SD    | C.S.% |
| 0                  |      | 747  | 761  | 754  | 747 | 761  | 9.6   | 1.3   |
| 0.5                | 185  | 757  | 748  | 564  | 185 | 757  | 327.9 | 58.2  |
| 1                  | 423  | 747  | 730  | 634  | 423 | 747  | 182.4 | 28.8  |
| 1.5                | 578  | 729  | 713  | 673  | 578 | 729  | 82.7  | 12.3  |
| 2                  | 669  | 710  | 703  | 694  | 669 | 710  | 21.9  | 3.2   |
| 2.5                | 712  | 698  | 703  | 704  | 698 | 712  | 7.0   | 1.0   |
| 3                  | 722  | 698  | 716  | 712  | 698 | 722  | 12.3  | 1.7   |
| 3.5                | 712  | 713  | 743  | 723  | 712 | 743  | 17.7  | 2.5   |
| 4                  | 693  | 745  | 784  | 741  | 693 | 784  | 45.9  | 6.2   |
| 4.5                | 674  | 793  | 837  | 768  | 674 | 837  | 84.6  | 11.0  |
| 5                  | 662  | 855  | 899  | 805  | 662 | 899  | 126.1 | 15.7  |
| 5.5                | 662  | 925  | 966  | 851  | 662 | 966  | 164.8 | 19.4  |
| 6                  | 678  | 998  | 1032 | 903  | 678 | 1032 | 195.1 | 21.6  |
| 6.5                | 711  | 1065 | 1090 | 955  | 711 | 1090 | 211.8 | 22.2  |
| 7                  | 760  | 1116 | 1131 | 1002 | 760 | 1131 | 210.1 | 21.0  |
| 7.5                | 822  | 1139 | 1145 | 1035 | 822 | 1145 | 185.1 | 17.9  |
| 8                  | 893  | 1121 | 1121 | 1045 | 893 | 1121 | 131.9 | 12.6  |
| 8.5                | 966  | 1045 | 1047 | 1019 | 966 | 1047 | 46.2  | 4.5   |
| 9                  | 1033 | 892  | 907  | 944  | 892 | 1033 | 76.9  | 8.1   |
| 9.5                | 1083 | 645  | 688  | 805  | 645 | 1083 | 241.3 | 30.0  |
| 10                 | 1104 | 281  | 371  | 585  | 281 | 1104 | 451.6 | 77.2  |

Once chilling requirements were fulfilled, heat accumulation begins until the start of the different phenological stages. The threshold temperature of 1 °C was the most accurate in our study since this showed the lowest CV% (Figure 3).



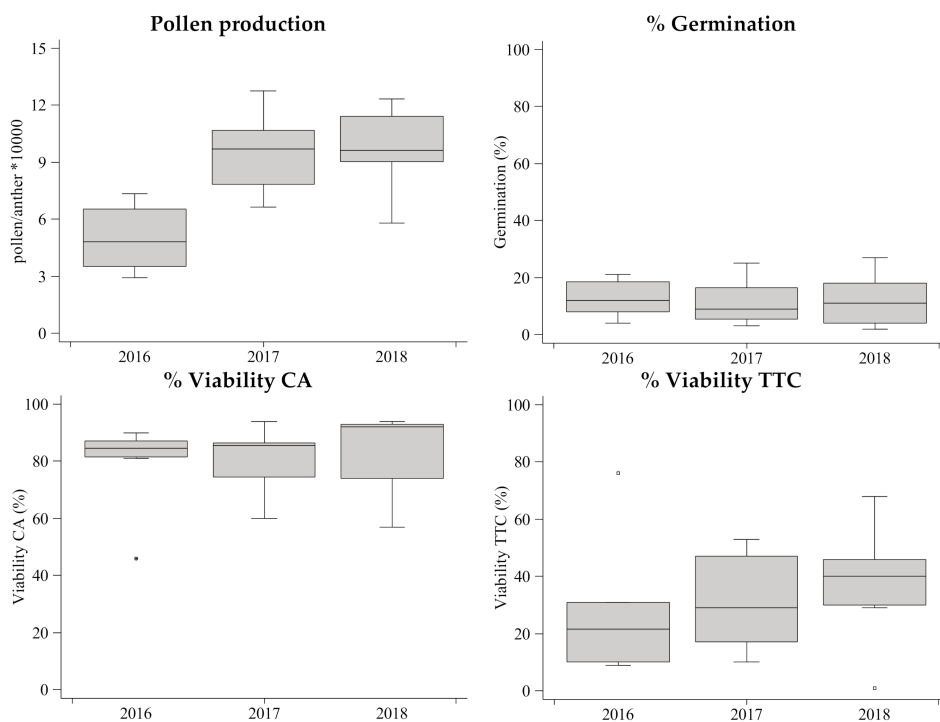
**Figure 3.** Heat requirements (GDD °C)—average values for the different phenological (S5: Stage 5; S6: Stage 6; S7: Stage 7; S8: Stage 8), considering 1 °C threshold temperatures (Th). In line with the standard deviation at each stage (SD).

On average, 399 GDD °C was necessary for stage 5 (inflorescence development) onset, 1139 GDD °C for the beginning of stage 6 (flowering), 1755 GDD °C for the start of stage 7 (fruit development), 2768 GDD °C for the beginning of stage 8 (fruit maturation), and 4463 GDD °C for harvest (Table S2, Figure 3). Considering the olive cycle from stage 5 (inflorescence development) to harvest, similar amounts of heat requirements were



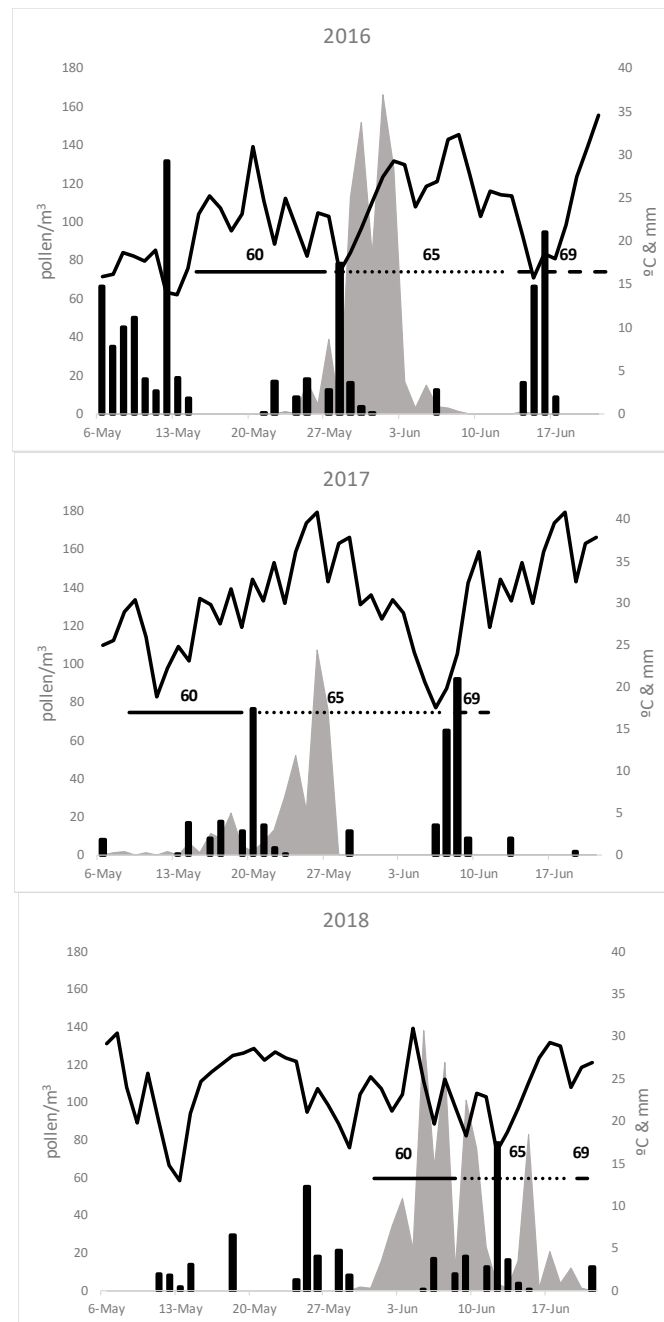
necessary during the study years: 4514, 4553 and 4323 GDD °C for 2016, 2017 and 2018, respectively.

Pollen production per anther during the studied years is shown in Figure 4, with an average of 82,589 pollen grains. Pollen production per anther was slightly higher during 2018 with an average of 99,089 pollen grains, ranging between 58,000 to 123,200 pollen, values, quite close to those obtained in 2017. The values registered in 2016 were lower with an average of 49,939 pollen grains per anther (Figure S2, Figure 4). In terms of pollen grain viability, the results obtained with the carmine acetic (CA) method were superior (81%) than those registered by the triphenyl-tetrazolium (TTC) (30%) in all the years of the study (Figure S2, Figure 4). The percentage of viability was higher in 2018, with an average of 84% and 38% for the CA and TTC methods, respectively. Pollen grains from 2016 showed the lowest viability rates, obtaining values between 80% and 28% with the CA and the TTC methods, respectively (Figure S2, Figure 4). In addition, the percentage for germination of the pollen tube showed rates of 12% on average. The percentage of germination was higher in 2016 and 2018 with 13% compared to the average value of 10% recorded in 2017 (Figure S2, Figure 4). No significant differences were detected between the years of the study by means of a Kruskal-Wallis test, with the exception of pollen production by anther (Table S3).



**Figure 4.** Pollen by anther, % of Germination, % of Viability with carmine acetic (CA) and triphenyltetrazolium (TTC) methods during the studied period. Median (black line), Box: 25–75%, Non-outlier (whisker) and outliers (point).

The presence of *Olea* pollen in the olive grove atmosphere was registered between the end of stage 5 (inflorescence development) and all of stage 6 (flowering), in which the highest amounts of pollen were recorded (Figure 5).



**Figure 5.** Dynamics *O. europaea* L. airborne pollen concentration (grey peaks) and daily meteorological parameters (maximum temperature in lines and rainfall in bars). In lines, duration of stage 6 (flowering) with the different phenophases (horizontal line): 60 beginning of flowering, 65 full flowering: 50% of flower open, and 69 end of flowering.

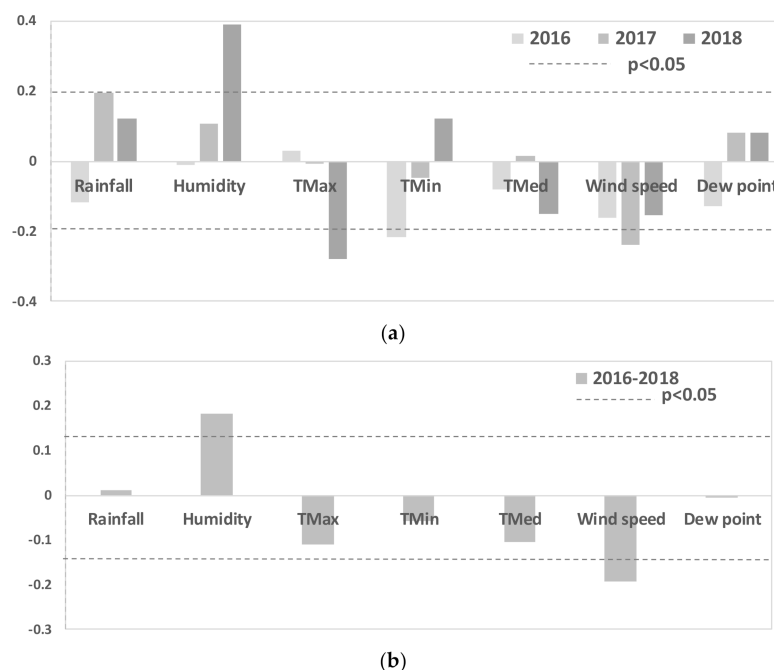
The main pollen season (MPS) started on average on 20th May and ended on 16th June with an average duration of 27 days (Table 2). The earliest start date was detected during 2017, three weeks earlier than in 2018. The end of the pollen season was constant between 14–20 June. The average annual pollen integral was 833 pollen grains ranging from 750 in 2016 to 947 in 2017. The maximum daily concentrations were registered in periods of high temperatures and without rainfall (Figure 4), with a peak value of 166 pollen/m<sup>3</sup> on 1 June 2016 (Table 2, Figure 5).



**Table 2.** Start and end date of the atmospheric *Olea* pollen season (days), total pollen amount (pollen/m<sup>3</sup>), peak day, concentration on peak day and total heat requirements for the start and end date of the pollen season. Average, standard deviation (S.D.) and standard deviation in percentage (C.S. %).

| Pollen Season                            |       | 2016    | 2017    | 2018    | Average | S.D.   | C.S.% |
|--|-------|---------|---------|---------|---------|--------|-------|
| Duration                                 | Start | 23-May  | 7-May   | 30-May  | 20-May  | 11.79  | 0.03  |
|  | End   | 14-June | 16-June | 20-June | 16-June | 3.06   | 0.01  |
| Total pollen grains                      |       | 750     | 947     | 801     | 833     | 102.25 | 12.28 |
| Peak day                                 |       | 1-June  | 30-May  | 5-June  | 1-June  | 3.06   | 0.01  |
| Polen peak day (pollen/m <sup>3</sup> )  |       | 166     | 135     | 138     | 146     | 17.10  | 11.68 |
| Growing Degreee Days (GDD) Pollen Season | Start | 1192    | 1053    | 1238    | 1161    | 96.24  | 8.29  |
|  | End   | 1558    | 1785    | 1581    | 1641    | 125.02 | 7.62  |

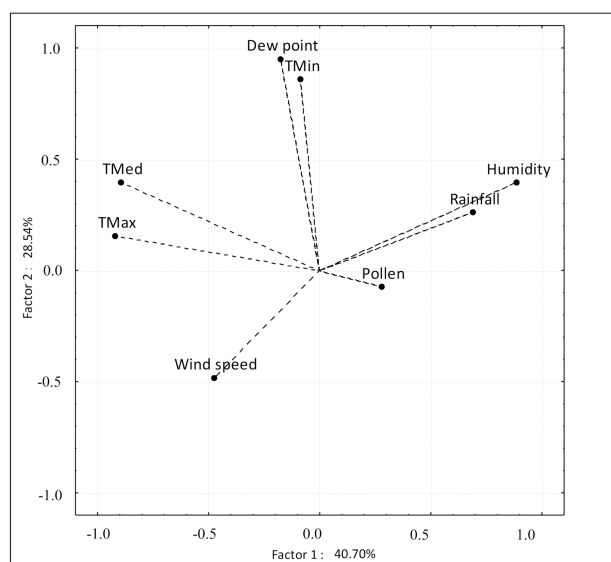
Regarding the correlations between atmospheric pollen and meteorological data, considering all the years of study, the Spearman correlation test showed significant positive correlations with humidity and a negative degree of association with wind speed and temperatures (Figure 6).



**Figure 6.** Spearman correlations between daily *Olea* pollen concentrations and the main meteorological variables considering all years of the study separately (a) and together (b).

Principal component analysis (PCA) was performed in order to better understand the influence of all weather variables in conjunction with airborne olive pollen concentrations. In our study, three components have been extracted, since they had eigenvalues greater than or equal to 1.0 and together they account for around 82.32% of the variability in the original data (Figure 7). Most of the variables have significant positive loadings in the first three Principal Components (PCs). In general, the three PCs were correlated as follows: Component 1 (average/maximum temperatures, rainfall and humidity), Component 2 (minimum temperature and dew point), Component 3 (pollen values). The results of the PCA show a high degree of positive association between the daily *Olea* pollen counts and water-related parameters (Figure 7).

|                  | Factor 1 | Factor 2 | Factor 3 |
|------------------|----------|----------|----------|
| Eigenvalue       | 3.26     | 2.28     | 1.05     |
| % Total variance | 40.70    | 28.54    | 13.08    |
| Cum. Eigenvalue  | 3.26     | 5.54     | 6.59     |
| Cumulative %     | 40.70    | 69.23    | 82.32    |
|                  | Factor 1 | Factor 2 | Factor 3 |
| Rainfall         | 0.689    | 0.259    | 0.206    |
| Humidity         | 0.887    | 0.394    | 0.003    |
| TMax             | -0.922   | 0.155    | -0.181   |
| TMin             | -0.087   | 0.859    | 0.237    |
| TMed             | -0.895   | 0.395    | -0.113   |
| Wind speed       | -0.476   | -0.483   | 0.343    |
| Dew point        | -0.176   | 0.951    | -0.090   |
| Pollen           | 0.278    | -0.074   | -0.881   |



**Figure 7.** Principal components and factor analysis (PCA). Rotation varimax. Extraction principal components for daily values for *Olea* pollen and main meteorological variables during the three years of study.

#### 4. Discussion

Climatic variations and forest soil conditions modulate floral phenology in olive trees and their phenological seasonality [58]. Temperature has been recognized as the factor that most affects crucial phenological stages such as flowering [2,44,59], or the completion of ecological events such as dormancy induction [1,60–63]. High temperatures are the main factor that increase the growth trend and the duration of the vegetative cycle in agronomical plants in temperate regions [59].

The base temperature of 2.5 °C recorded the lower standard deviation coefficient in the studied area with an amount of 704 CH following the V.A. method [42]. Several studies have been carried out on this topic in recent years, most of which indicate a higher base temperature of between 9.5 and 12.5 °C in southern Italy [64] and 12 °C in southern Spain [65]. Most of the research on the thermal needs of olive trees has been carried out throughout the Mediterranean basin. The location of the olive grove sampled at the limits between the Eurosiberian and Mediterranean bioclimatic areas could explain the important variations observed in our study. Several authors have pointed out that variations in thermal requirements are related to the different bioclimatic conditions of olive groves [1,31,66–68].

Once the chilling requirements are met, the crown buds remain in temporary suspension, and their visible growth status is controlled by environmental factors (ecodormancy) [69]. Our study also detected a low base temperature of 1 °C for heat accumulation. The observed threshold is notably lower than that indicated by different authors in order southern latitudinal areas, 10–13 °C in California [70], 10–12.5 °C in southern Spain [1,44], 9–9.7 °C in Portugal [71] and 7 °C in norther Mediterranean areas of Spain [33]. Threshold temperature is strongly influenced by geographic conditions, altitude and latitude showing an inverse relationship with base temperature [1,2,72]. In addition, different type of species or cultivars can also affect the base temperature determination [73–75]. Taking into account this threshold, an average of 1139 GDD was required to reach flowering, a higher value than the 180–380 GDD indicated for southern Spain [1] or another Mediterranean location in Northern Spain with 526 GDD [33]. Considering the olive cycle from stage 5 (inflorescence development) to harvest, similar amounts of heat requirements were necessary during the years of study, 4514, 4553 and 4323 GDD for 2016, 2017 and 2018, respectively. In general, there are no significant differences for the amount of GDD needed to reach each of the stages with the sole exception of the beginning of stage 5, for which a lower value of 243 GDD was obtained in 2016 compared to the 410–544 GDD for the years 2017 and 2018 respectively.

To accurately evaluate the suitability of the olive cultivars in a given area, studies on their reproductive biology are necessary, considering pollen production, viability and germination capacity [76]. In the sampled olive trees, an average of 82,589 pollen grains per anther was recorded during the three years, with values ranging between 35,000 and 127,000 pollen grains. Similar values were reported by other authors in Mediterranean olive groves indicating a mean value of 84,333 in four different cultivars [77], or average values of 79,802 pollen grains by anther in 13 different olive varieties [21]. The proportion of staminate flowers and the success of pollen viability and germination in the olive tree are other key factors for successful fertilization rates that vary depending on the cultivar, climatic conditions, and growing area [76]. The mean values of pollen variability detected in our study ranged from 30% viability according to the TTC method and 81% viability according to the CA method. Ferrara et al. [21] indicated similar values, while Mazzeo et al. [78] observed higher viability percentages of around 90% by the CA method and 58% by the FDA method (Fluorescein diacetate) in Italian vineyards.

Furthermore, the fertilization processes were also influenced by the precise germination rates of the pollen grains. Our study detected low germination values of around 12%, compared to the 48% obtained by Mazzeo et al. [78] in Italy, the 20.6–64.9% by Pinney and Polito [63] or the 35.5% obtained in Australia by Wu et al. [79]. Variations in the extent of olive pollen germination were consequence of the cultivars, orchards, and germination media used, oscillating between 2.1% and 31.6% [63,76,78–80]. Significant differences in viability and germination have even been reported within clones of the same cultivar (Arbequina and Leccino) [80]. The level of pistil abortion and the success of pollen germination are genetically determined and are likely to be an evolutionary adaptation to redistribute available resources [76]. Therefore, the differences among cultivars and years of study in terms of pollen viability can be attributed to physiological and environmental factors, but genetic influence might also play a major role [78]. The beneficial effect of foliar treatments with Boron immediately one week before anthesis has been reported, increasing pollen germination rates [17], possibly by positively affecting the fertilization process and the subsequent plant source–sink relations linked to fruitlet retention. Studies conducted by Fernández-González et al. [81] indicated a capacity for high expression in olive pollen grains of proteins involved in the germination processes in years with lower pollen production in order to ensure an adequate pollen germination and tube elongation for a successful fertilization process.

The potential amount of olive harvesting of a given variety is related to the presence of pollen in the olive grove atmosphere during the flowering stage [22,82]. Both the beginning of the *Olea* pollen season and the flowering stage depend on the temperature in the previous

months [68], as a consequence of the fact that spring temperatures strongly limit the effect of winter temperatures on the processes of flower induction or dormancy [28]. Our results detected some variations between the onset and the duration of the MPS during the study years with a mean start date of 19th May and a mean duration of 29 days. The start of the pollen season in the year 2017 occurred a month early, resulting in a longer pollen season. These values differ slightly from those indicated by other authors, since in general the onset occurs a little later with a slightly shorter MPS duration. In southern Spain, advanced average onset data were observed until April or early May and an average duration of around 35 days [1,83,84]. The beginning of the MPS at the end of March and average duration of 89 days were noted for Morocco [60]. In addition, an average start date in mid-May and a higher average duration of 48 days was detected in Mediterranean areas closest to Northern Spain locations [2,33]. These fluctuations are a consequence of the biogeographical and climatic differences between the areas, mainly altitude, temperature, and rainfall in the previous months [1,31,60].

Regarding the pollen concentrations in the olive grove atmosphere, maximum daily concentrations ranging between 138–166 pollen/m<sup>3</sup> and annual pollen integral of 833 pollen grains were observed for the study period. The low pollen concentrations were due to the northerly location of the grove in a bioclimatic transition zone between the Eurosiberian and the Mediterranean areas, at the limit of olive tree distribution. Studies conducted by Garrido et al. [32] in other Northern Mediterranean areas reported annual totals of 3500 pollen grains with peaks of around 600 pollen/m<sup>3</sup>. In the African Mediterranean basin, maximum daily pollen peaks of 183 pollen grains/m<sup>3</sup> and a total amount of 3089 pollen grains were observed [60]. In the main olive-producing areas of the southern Spain higher values of 44,024 pollen throughout the year and maximum daily concentrations of 4795 pollen grains/m<sup>3</sup> were detected [31,83]. Extensive land use for olive cultivation and diverse meteorological conditions could be considered as the causes of the aforementioned quantitative differences. A positive correlation was observed in our study between pollen and rainfall or humidity, and negative for wind speed. This behaviour is opposite to that indicated by other authors in the Mediterranean bioclimatic areas [31,32,60,83,85,86]. The negative degree of association with wind speed indicates that the *Olea* pollen observed in the atmosphere of the olive grove is mainly released in the atmosphere of the cultivar studied. Hybrid Single Particle Lagrangian Integrated Trajectory Model (HYSPLIT) models carried out in southern Mediterranean areas of North-western Spain located 150 Km from our study area observed a long-range transport for olive pollen grains, since the pollen peaks during the days prior to the flowering stage came from the extensive olive groves of Northern Portugal which bloom a few days in advance [32].

## 5. Conclusions

The vegetative period of the olive tree in the study area lasted for an average of 259 days. The important phenological stage 6 of flowering was the shortest, from the second tenth of May to mid-June. To predict the onset of flowering, a thermal time model was used in order to quantify the chill requirements, and growing degree-days was applied to determine the heat requirement. An average of 704 CH with a threshold of 2.5 °C were required to overcome the chilling period, 1139 GDD for the beginning of flowering, and 4463 GDD °C for harvest.

To accurately evaluate the suitability of the olive cultivars in a given area, studies of their reproductive biology are essential. The pollen production per anther was 82,589 pollen grains, with a rate of 81% viability and 12% germination of the pollen tube. The presence of *Olea* pollen in the olive grove atmosphere was registered between the end of stage 5 (inflorescence development) and all of stage 6 (flowering), in which the highest amounts of pollen were recorded. The olive MPS started on average on May 20th and ended on June 16th with an average duration of 27 days and an annual pollen integral of 833 pollen grains. The low pollen concentrations could be a consequence of the Northern location of

the forest, in a bioclimatic transition zone between the Eurosiberian and the Mediterranean areas, at the limit of olive tree distribution.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/1999-4907/12/2/204/s1>, Table S1: Mean, maximum and minimum dates and length (duration in days) of the different sub stages during the years of the study. (S.D. Standard deviation; C.S.% Coefficient of Standard deviation in percentage). Table S2: Heat requirements (GDD) for the different phenological stages (S5: Stage 5; S6: Stage 6; S7: Stage 7; S8: Stage 8), considering different threshold temperatures (Th). Mean, standard deviation (S.D.) and coefficient of Standard deviation in percentage (C.S.%). Figure S1: Pollen by anther, % of Germination and % of Viability with CA and TTC methods during the studied period with the mean, maximum, minimum, standard deviation (S.D.) and coefficient of standard variation in percentage (C.S.%).

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