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Analysis of the Variability of Floral and Pollen Traits in Apple Cultivars—Selecting Suitable Pollen Donors for Cider Apple Orchards

Alvaro Delgado ^{1,*} , Muriel Quinet ²  and Enrique Dapena ¹ 

¹ Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Apdo.13, E-33300 Villaviciosa, Asturias, Spain; edapena@serida.org

² Earth and Life Institute-Agronomy, Université Catholique de Louvain, Croix du Sud 4-5, Box L7 07 13, 1348 Louvain-la-Neuve, Belgium; Muriel.Quinet@uclouvain.be

* Correspondence: alvaro.delgadodelgado@serida.org

Abstract: Most apple trees (*Malus domestica* Borkh.) are self-incompatible and fruit yield depends on cross-pollination between genetically compatible cultivars with synchronous flowering. Flowering intensity can vary strongly among years due to the biennial bearing habit of the cultivars. The knowledge of the phenological stages and floral and pollen characteristics is essential to select suitable pollen donors. We evaluated the phenotypic variability of flowering-related traits (i.e., flowering phenology, flowering intensity, pollen production and pollen quality) in 45 apple cultivars over two successive flowering seasons. Large phenotypic variability was found among the studied cultivars indicating that the local germplasm collection provides a good source of genetic and phenotypic diversity. However, low correlations were observed between floral biology traits and, consequently, the improvement in one trait seems not to affect other traits. Some of the cultivars such as ‘Perurico’ and ‘Raxila Dulce’ regularly produced copious amounts of high-quality pollen which can improve the pollen load dispersion leading to a most effective pollination process. We did not identify statistically significant correlations between pollen attributes and the biennial bearing phenomenon. The large variation in bloom dates from year-to-year observed under a typical Oceanic climate makes it advisable to combine cultivars in new plantings.

Keywords: *Malus domestica*; blooming time; pollen quality; pollen production; ‘on’ and ‘off’ years; phenotypic variation



Citation: Delgado, A.; Quinet, M.; Dapena, E. Analysis of the Variability of Floral and Pollen Traits in Apple Cultivars—Selecting Suitable Pollen Donors for Cider Apple Orchards. *Agronomy* **2021**, *11*, 1717. <https://doi.org/10.3390/agronomy11091717>

Agronomy **2021**, *11*, 1717. <https://doi.org/10.3390/agronomy11091717>

Academic Editor: Pilar S. Testillano

Received: 26 July 2021

Accepted: 25 August 2021

Published: 28 August 2021

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1. Introduction

Pollination is a key event in plant reproduction which consists in the transfer of pollen grains from anthers to stigmas in order to fertilize the ovules and begin the development of seeds and fruits [1]. Apple (*Malus domestica* Borkh.) is known as a gametophytic self-incompatible flowering tree [2] and yield relies on the successful cross-pollination between at least two cross-compatible cultivars which flower synchronously [3,4].

The quantity and quality of apple production is significantly affected by the effectiveness of the pollination process [5,6]. Apart from the fundamental requirement for fruit set and tree productivity, the presence of different pollen sources has a favorable impact on fruit quality parameters such as size, shape and organic matter [5,7–10]. Apple trees are heavily dependent on insect pollination and the contribution of pollinators is also essential for obtaining consistent yields [3,5,6].

To avoid pollen limitation in commercial orchards, either pollen donor trees (i.e., “pollinizers”) or cultivar mixtures are planted in the same orchard [11,12]. Garratt et al. [5] and Quinet and Jacquemart [13] stated that the improvement in the density and arrangement of pollinating cultivars in the orchard promotes the share of pollinators carrying viable pollen, eventually enhancing fruit and seed set.

Flowering is one of the main processes leading to crop productivity [14] in fruit trees, therefore, their phenotypic study has practical implications for breeding purposes. To the extent that fruit yield and quality is primarily the result of the flower quantity and quality [15], the modification and improvement of relevant floral biology traits is expected to play a major role in the process of obtaining a more sustainable productivity. Since apple orchards require several years before new plantings become profitable, the selection of cultivars is vitally important. For the characterization of pollen donor plants, also known as pollinizers, it is essential to evaluate a variety of phenotypic traits such as floral overlap with productive varieties, bloom intensity, pollen production and pollen quality. Flowering intensity and pollen production are important traits to measure the pollination ability of any cultivar due to that a higher number of pollen grains can improve the success of ovule fertilization and, consequently, the fruit set of commercial trees [16]. The production of pollen is cultivar-specific [17] and it is primarily determined by the pollen quantity per anther. Additionally, the quality of the pollen characterized by pollen viability and/or pollen germination must also be considered with the goal of ensuring economically acceptable yields. Pollen germination studies on apple trees have shown wide variability for the same variety depending on the trees' location and the laboratory assessment [4], but it has been proven that triploid cultivars exhibit lower pollen germination rates than diploid cultivars [18,19].

Apples are an economically important agricultural product in Asturias. This region, situated in north-western Spain, has a long tradition in cider-making and inherently linked to this tradition there is a high diversity of local apple cultivars. The Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) currently maintains 800 apple accessions, of which approximately 500 are local accessions [20]. Over the last three decades, the bulk of the cider apple orchards planted in Asturias are composed of between four and six different cultivars. These cultivars are part of the 22 cultivars initially included in the quality label "Protected Designation of Origin (PDO) Asturian Cider." Recently, the PDO regulatory board endorsed 54 new apple varieties, including 18 new varieties selected by the SERIDA breeding program, which are expected to be planted in Asturias and other regions over the next few years. Most of the cider apple orchards in this region are planted on semi-dwarfing rootstocks with a tree density of around 650 trees/ha. This system contrasts with high-density plantings of many apple growing regions where solid monovarietal blocks were widely implemented. In a recent study, Martínez-Sastre et al. [21] did not find a deficit of pollinators in most of the Asturian apple orchards. Hence, the maximum level of pollination in these farms may potentially be achieved by ensuring the presence of large quantities of compatible pollen and facilitating cross-pollination accomplished by pollinators [5,13,22].

On the other hand, the study region relies on local cider cultivars which exhibit different degrees of biennial or alternate bearing [23,24]. Biennial bearing is a common problem in apple trees where the excessive number of fruits in 'on' years trigger a competition between fruit formation and flower initiation, resulting in low or non-existent yields in 'off' years [25–27]. At the regional level, this situation causes problems in the supply of apples to the cider industries in 'off' years and increases the risks of disruptions in the commercialization during 'on' years. Mazzeo et al. [28] showed that olive trees produce less pollen grains but with a significantly higher viability in 'off' years with the aim to maximize the fertilization of the ovules. The relationship between the quantity and quality of pollen grains affected by the 'on' and 'off' year has not been well studied in apples.

The main aim of this study was to characterize and describe the variability of some flowering-related traits among 45 apple cultivars in order to optimize their use for pollination purposes and also to ascertain if the knowledge of these floral and pollen characteristics can support new breeding strategies. More specifically, using a range of replicated field and laboratory experiments, this study determined: (1) periods of bloom overlap between cultivars; (2) flowering intensity and pollen production and (3) viability and germination of pollen grains. This work also raised the following questions: (i) is the quality and number

of pollen grains affected in an ‘off’ year with respect to an ‘on’ year in apple trees? and (ii) which cultivars can better meet the pollen donor requirements?

2. Materials and Methods

2.1. Site Description and Plant Material

The study was carried out in Villaviciosa, Asturias, north-western Spain (43°28′ N, 5°26′ W, 10 m above sea level). Forty-five apple cultivars (Table S1) were evaluated in three nearby experimental plots belonging to SERIDA (within a radius of approximately 150 m). In 2018 and 2019, twenty-two cultivars included in the quality label “Protected Designation of Origin Asturian Cider” since 2002 [23] were evaluated in a single block of three trees of each cultivar within a 10-year-old orchard. In 2019 and 2020, we observed a block of two trees for each cultivar in a 9-year-old orchard comprising twenty-one cultivars from the SERIDA apple breeding program (eighteen cider apple cultivars and three dessert apple cultivars). Fourteen of these cultivars were recently registered as protected varieties and the remaining seven are currently in the process of being registered. The dessert apple cultivar ‘Granny Smith’ and the crab apple *Malus floribunda* Siebold ex Van Houtte clone 821 grown in an 18-year-old repository orchard were included as international references. All plots consisted of semi-dwarf trees grown on vertical axis. Trees were grafted onto M.7 rootstock and their planting distance was 5.5 m × 2.5 m, apart from *Malus floribunda* 821 and ‘Granny Smith’ trees which were grafted onto PI.80 rootstock and planted with a distance of 4.75 m × 1.75 m. The research orchards have similar soil conditions, are managed under organic certification standards and trees are normally pruned every year. Each cultivar was evaluated over a period of two successive flowering seasons, including both an ‘on’ and an ‘off’ year except for *Malus floribunda* 821, ‘Blanquina’, ‘Meana’ and ‘Verdialona’ which were assessed only in one season.

2.2. Flowering Dates, Flowering Duration and Bloom Synchronization

Flowering phenology observations were carried out twice a week according to the international BBCH code for pome fruit [29] from green tip (stage 53) until petal fall (stage 67). Flowering time was established as the moment when trees reached stage 65 of the BBCH (full bloom, approximately 50% of flowers open). The duration of flowering was calculated as the period (days) between the phenological growth stage 61 of the BBCH code (first bloom, approximately 10% of flowers open) and petal fall. Bloom synchronization was determined based on the overlapping periods between cultivars.

2.3. Flowering Intensity and Pollen Production

The total number of inflorescences on each tree for all cultivars was counted using a tally counter. Accurate counts get more complicated after the flowers start to open [30], consequently, the total number of inflorescences for each cultivar was recorded between the phenological stages 61 and 65 of the BBCH code [29]. This approach was taken instead of counting the number of inflorescences on typical branches [31] due to the selected cultivars exhibiting pronounced differences in vigor, tree architecture and fruit-bearing habits [23,24]. The trunk circumference was measured at 40 cm height from the ground, approximately 20 cm above the graft union, and density of blossoms was converted to flowers per square centimeter of trunk cross-sectional area (TCSA) [32]. The number of flowers per inflorescence was evaluated by counting the number of flowers per inflorescence in 10 typical inflorescences per tree in the studied block. The number of anthers per flower in each cultivar was determined by counting the number of anthers in ten flowers randomly picked at balloon stage. With the intention of determining the number of pollen grains in one flower, one sample of 20 anthers from each cultivar was prepared by placing all the anthers in a 2 ml Eppendorf tube and letting them to dehisce in a growth chamber at 21 °C for 48 h. One milliliter of aqueous eosin solution (C.I. + PO₄^{3−}) was added to the dried anthers and samples were shaken on Vortex for 30 s just before 0.2 mL from the solution was applied to a Malassez hemocytometer (adapted from Bieniasz et al. [33]). Two

counts from the same tube were performed with the Malassez hemocytometer. Pollen grains were counted using a Nikon Eclipse 50i compound microscope at 10× magnification (Figure S1a) and the number of pollen grains in one anther was calculated by dividing the total amount of pollen grains in 20 anthers by 20.

2.4. Evaluation of Pollen Quality

Pollen quality was evaluated using two different parameters, pollen viability and pollen germination. A total of 30 flowers at balloon stage from all the trees in the experimental block were sampled and anthers were removed. Pollen from these anthers was left to dehisce in Petri dishes at 21 °C for 48 h. Pollen viability was assessed using Iodine Potassium Iodide (IKI) staining technique (1 g potassium iodide (KI) + 0.5 g iodine (I) dissolved in 100 mL distilled water). Pollen was spread in a microscope slide, mixed with 120 µL of the solution and covered with a coverslip. Counts were made using a Nikon Eclipse 50i compound microscope ten minutes after pollen was placed on IKI solution. One hundred pollen grains in four different areas of the same preparation were observed. Pollen grains stained dark brown were scored as viable and unstained grains were counted as non-viable (Figure S1b). Pollen viability tests were not conducted in 2018. On the same day as pollen viability tests were carried out, pollen from the same Petri dishes was used to determine the germination of pollen grains. Pollen germination was evaluated *in vitro* by dusting the pollen on concentrated agar (1.5%) containing 10% sucrose and boric acid (240 mg/L). After 24 h at 21 °C, pollen germination was measured using an Olympus SZX12 stereomicroscope (Olympus Corporation, Tokyo, Japan). Four different subsamples of one hundred pollen grains from the same agar plate were observed and pollen germination percentage was calculated. Pollen grains were considered germinated when the length of a pollen tube exceeded its diameter ([34]; Figure S1c).

2.5. Alternate Bearing Tendency and Its Influence on Pollen Properties

Return bloom was calculated according to the number of inflorescences/TCSA cm² on two successive growing seasons [35]. The growing season with the largest number of inflorescences was considered the ‘on’ year and this information was used to determine if there is a correlation between pollen quantity and quality in an ‘off’ year with respect to an ‘on’ year. If at least one of the trees in the studied block showed a different biennial bearing pattern, data from this cultivar was discarded for further analysis. Hence, twenty-four cultivars which showed a clear biennial bearing pattern during these two seasons were selected for the analysis.

2.6. Statistical Analysis

Prior to all statistical analyses, the assumption of normality was investigated using the Shapiro–Wilk test. Pollen germination and pollen viability rates were transformed into arcsine square root values. Duration of flowering was analyzed after log 10 transformation. One-way ANOVA was used to investigate the differences among cultivars and also to determine changes in pollen properties between ‘on’ and ‘off’ years. Means were compared with Duncan’s Multiple Range Test at $p < 0.05$. Correlation coefficients between floral and pollen characteristics were determined using Pearson’s correlation coefficient using individual values (year and cultivar) for each variable. All analyses and figures were produced in the R programming environment (version 4.0.4; [36]).

3. Results

3.1. Phenological Observations, Duration of Flowering and Flowering Overlap between Cultivars

Full bloom was first observed in *Malus floribunda* 821 on March 24th. Excluding this early flowering crab apple species, the two-year mean bloom date ranged from April 13th (‘Colorá Amarga’) to May 20th (‘Raxao’). The mean bloom date for the set of apple cultivars was recorded on May 8th (season 2017/2018), April 23rd (season 2018/2019) and April 30th (season 2019/2020). Pronounced year-to-year variation in flowering dates

was observed between flowering seasons for most of the evaluated cultivars, although the order of flowering among cultivars was similar in both years (Figure S2). Approximately 40% of the cultivars exhibited similar flowering time and flowered within the same week. Among the newly registered cultivars (Table S1), we found a lower proportion of late-flowering cultivars, and only the cultivar ‘Raxarega’ belonged to the very late-flowering group (Figure S2).

The average duration of flowering ranged between 7 days in cultivars ‘Verdialona’ and ‘X9406-11’ and 16 days in cv. ‘Raxona Ácida’. The cultivars ‘Raxona Ácida’ and ‘Raxona Dulce’ exhibited a mean blooming duration of 16 days with also a slight variation between years in the length of the period. For the set of studied cultivars, bloom duration varied from 10 to 15 days in 2017/2018, from 8 to 20 days in 2018/2019 and from 7 to 14 days in 2019/2020. Less than one quarter of the cultivars showed a two-year average duration shorter than 10 days (Table 1).

Table 1. Flowering time, flowering duration, flowering intensity (number of inflorescences/cm² of trunk cross-sectional area) and floral characterization of 44 domesticated apple cultivars and *Malus floribunda* 821 in Villaviciosa (north-western Spain) in two successive years (i.e., 2018–2019 or 2019–2020). Data from two successive flowering seasons was pooled to obtain a unique value for each cultivar and variable. Two-year mean values are accompanied by the standard deviation. ANOVA results using the year and cultivar as factors are shown.

Cultivar	Flowering Date	Duration of Flowering	Inflorescences/TCSA (cm ²)	Number of Flowers/Inflorescence	Number of Anthers/Flower
Blanquina	28 April	12 ± 2.83	4.88 ± 3.13	5.7 ± 0.59	18.4 ± 1.58
Carrió	2 May	10.5 ± 0.71	5.22 ± 7.54	5.7 ± 0.54	19.05 ± 0.95
Clara	18 April	11.5 ± 3.53	8.99 ± 2.20	6.08 ± 0.63	17.6 ± 1.43
Collaos	4 May	10.5 ± 0.70	10.35 ± 4.50	5.46 ± 0.65	18.2 ± 1.16
Cladurina	17 April	12.5 ± 2.12	13.2 ± 7.55	5.75 ± 0.63	18.15 ± 1.72
Cladurina	15 April	12.5 ± 0.71	8.3 ± 9.71	5.45 ± 0.69	18.55 ± 1.66
Amargo-Ácida	7 May	10.5 ± 0.70	19.2 ± 4.81	5.83 ± 0.66	19.1 ± 1.52
Colladina	13 April	12 ± 2.83	7.9 ± 2.93	5.57 ± 0.50	18.6 ± 0.99
Colorá Amarga	18 April	11.5 ± 2.12	10.99 ± 2.47	5.97 ± 0.50	18.45 ± 1.32
Coloradona	1 May	9.5 ± 3.53	12.27 ± 10.74	5.42 ± 0.76	19.65 ± 1.53
De la Riega	18 April	12.5 ± 1.41	17.7 ± 9.90	5.55 ± 0.69	17.3 ± 1.15
Durcolorá	8 May	10.5 ± 0.71	4.45 ± 6.52	4.17 ± 0.77	19.67 ± 0.82
Durona de Tresali	1 May	8.5 ± 2.12	8.36 ± 9.89	5.52 ± 0.68	19.2 ± 1.55
Ernestina	6 May	10 ± 0	5.93 ± 6.71	5.68 ± 0.69	19.25 ± 1.17
Fuentes	17 April	7.5 ± 0.71	6.18 ± 1.26	5.73 ± 0.51	19.15 ± 1.07
Granny Smith	13 May	10 ± 0	6.78 ± 4.06	5.75 ± 0.58	19.05 ± 1.28
Limón Montés	24 March	11	27.67 ± 17.62	7.15 ± 0.67	20.8 ± 1.48
<i>Malus floribunda</i> 821	10 May	14	7.98 ± 4.69	5.7 ± 0.69	19.4 ± 1.18
Meana	2 May	12 ± 2.82	8.25 ± 7.40	5.3 ± 0.77	19.1 ± 1.41
Panquerina	25 April	9.5 ± 0.71	8.22 ± 7.27	5.8 ± 0.71	19.6 ± 1.05
Perezosa	11 May	12.5 ± 2.12	9.20 ± 7.23	5.63 ± 0.54	18.75 ± 0.97
Perico	26 April	9 ± 1.41	15.21 ± 10.99	5.73 ± 0.66	18.8 ± 1.06
Perurico	27 April	10.5 ± 4.95	13.95 ± 13.11	5.68 ± 0.63	17.85 ± 1.31
Perurico Precoz	13 May	12 ± 2.83	10.00 ± 8.20	5.77 ± 0.50	18.55 ± 1.28
Prieta	20 May	8.5 ± 2.12	7.8 ± 5.56	5 ± 0.58	19.15 ± 1.19
Raxao	13 May	12 ± 2.83	6.93 ± 3.81	5.43 ± 0.49	18.05 ± 1.15
Raxarega	28 April	9 ± 1.41	7.2 ± 4.02	5.95 ± 0.51	18.55 ± 1.36
Raxila Ácida	18 April	12.5 ± 2.12	12.09 ± 4.11	6.25 ± 0.44	19.05 ± 1.09
Raxila Dulce	18 April	9 ± 2.82	20.3 ± 2.69	5.78 ± 0.55	19.15 ± 0.88
Raxila Rayada	1 May	12 ± 2.80	18.5 ± 10.39	5.53 ± 0.66	19.05 ± 0.94
Raxina Ácida	27 April	12.5 ± 3.5	11.9 ± 1.73	4.88 ± 0.58	18.55 ± 0.88
Raxina Amarga	29 April	9 ± 2.83	5.9 ± 2.91	5.1 ± 0.52	19.05 ± 1.10
Raxina Dulce	28 April	11.5 ± 0.71	11.19 ± 4.81	5.05 ± 0.39	18.05 ± 1.87
Raxina Marelo	5 May	16 ± 7.07	15.9 ± 9.69	5.4 ± 0.50	19 ± 1.03
Raxona Ácida	3 May	15.5 ± 2.12	12.13 ± 5.14	5.58 ± 0.61	18 ± 1.30
Raxona Dulce	8 May	10.5 ± 3.54	7.31 ± 8.22	5.65 ± 0.64	19.75 ± 1.52
Regona	17 April	11 ± 0	13.8 ± 4.48	5.95 ± 0.58	19.65 ± 0.93
Rosadona	1 May	11 ± 1.41	11.45 ± 11.09	5.28 ± 0.76	16.7 ± 2.00
San Roqueña	6 May	11 ± 0	6 ± 5.64	5.45 ± 0.58	18.45 ± 1.36
Solarina	4 May	10.5 ± 4.95	4.8 ± 3.71	5.64 ± 0.64	18.15 ± 1.23
Teórica					

Table 1. *Cont.*

Cultivar	Flowering Date	Duration of Flowering	Inflorescences/TCSA (cm ²)	Number of Flowers/Inflorescence	Number of Anthers/Flower
Verdialona	7 May	7	3.96 ± 5.37	5.2 ± 0.72	19.5 ± 1.17
X9406-49	22 April	12 ± 2.83	19.14 ± 13.11	5.43 ± 0.64	18.15 ± 1.56
X9406-57	17 April	12.5 ± 2.12	12.3 ± 7.98	5.25 ± 0.59	18.7 ± 1.39
X9406-11	20 April	7 ± 0	17.5 ± 8.24	5.6 ± 0.50	17.7 ± 1.52
Xuanina	2 May	10.5 ± 4.95	10.94 ± 5.06	5 ± 0.68	18.9 ± 0.91
ANOVA					
Cultivar		F _{44,41} = 1.14, <i>p</i> = 0.35	F _{44,162} = 2.86, <i>p</i> < 0.001	F _{44,1693} = 15.78, <i>p</i> < 0.001	F _{44,815} = 1.79, <i>p</i> = 0.035
Year		F _{1,41} = 4.8, <i>p</i> = 0.02	F _{1,162} = 4.98, <i>p</i> = 0.028	F _{1,1693} = 4.30, <i>p</i> = 0.014	F _{1,815} = 0.74, <i>p</i> = 0.48

3.2. Phenotypic Evaluation of Floral and Pollen Traits

The number of flowers per inflorescence varied among cultivars (Table 1). The mean number across the studied cultivars was 5.6. The largest and the lowest mean number of flowers per inflorescence were found in *Malus floribunda* 821 (7.2) and ‘Durona de Tresali’ (4.2), respectively. Large variability was observed especially for the number of blossom clusters/TCSA (cm²) (Table 1). The cultivar ‘Raxila Rayada’ showed the highest two-year average value (20.3) whereas the minimum number was recorded in ‘Verdialona’ (4.1). The number of anthers per flower ranged from a minimum of 16.7 (‘San Roqueña’) to a maximum of 20.8 (*Malus Floribunda* 821), with most of the cultivars having between 18 and 20 anthers per flower (Table 1). The number of pollen grains per anther showed a wide variability among cultivars ($F_{44,127} = 2.29$, $p = 0.005$; Figure 1) and this value ranged from 1319 in ‘Cladurina Amargo-Ácida’ to 8056 in ‘Raxila Dulce’.

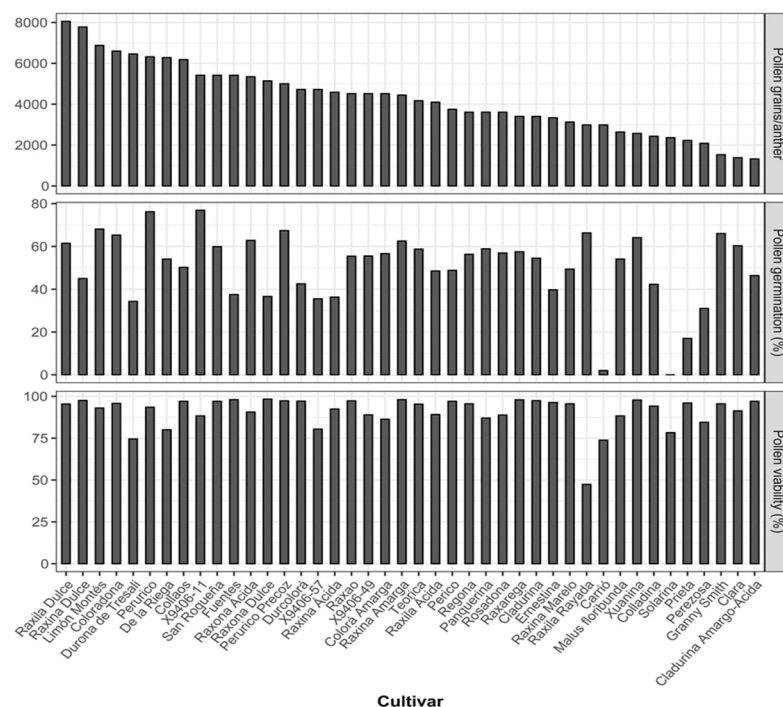


Figure 1. Means of pollen grains per anther, pollen germination (%) and pollen viability (%) of 41 apple cultivars in Villaviciosa (north-western Spain) across two successive years (i.e., 2018–2019 or 2019–2020).

All cultivars with the only exception of 'Raxila Rayada' showed a pollen viability value above 70% (Figure 1). The highest and the lowest pollen viability estimates were found in 'Raxona Dulce' (98.3%) and 'Raxila Rayada' (47.4%), respectively. Pollen germination

differed considerably among cultivars ($F_{44,256} = 18.90$, $p < 0.001$; Figure 1). The lowest mean germination rates were detected in ‘Solarina’ and ‘Carrió’ which are triploid cultivars (Dapena, personal communication). Only considering diploid cultivars, the maximum germination percentage (76.9%) was found in the accession ‘X9406-11’ whereas the lowest value was observed in cv. ‘Prieta’ (17%). Pollen germination and viability percentages in both years were similar for each cultivar, although a greater level of variation among cultivars was found in pollen germination (coefficient of variation; CV = 34.5) than pollen viability estimates (CV = 12.3; Figure 1). Pollen viability was higher than the pollen germination in all but one of the studied cultivars.

3.3. Effects of Biennial Bearing on Floral and Pollen Traits

Important differences in the density of blossoms between two successive growing seasons were observed among the examined apple cultivars. The number of inflorescences/TCSA cm² in an ‘off’ year was generally lower in the traditional apple cultivars than in the new releases obtained by the breeding program. Among the twenty-four cultivars used for this study, the return bloom percentages ranged from 0.4 (‘Ernestina’) to 84.2 (‘Raxina Amarga’; Table S2).

Biennial bearing pattern showed a statistically significant effect on the duration of flowering ($F_{1,23} = 8.11$, $p = 0.009$). The average flowering duration in ‘on’ and ‘off’ years were 11.8 and 9.8 days, respectively. We did not find statistically significant differences between ‘on’ and ‘off’ years for the other flowering traits analyzed in this work. Pollen viability, pollen germination and pollen production showed almost the same values during both seasons (Figure 2). Although no general effect of biennial bearing was observed on these traits, significant differences were identified for cv. ‘Limón Montés’ which showed higher values during the ‘off’ year for pollen germination ($F_{1,7} = 25.5$, $p < 0.001$) and pollen production ($F_{1,3} = 13.03$, $p = 0.011$).

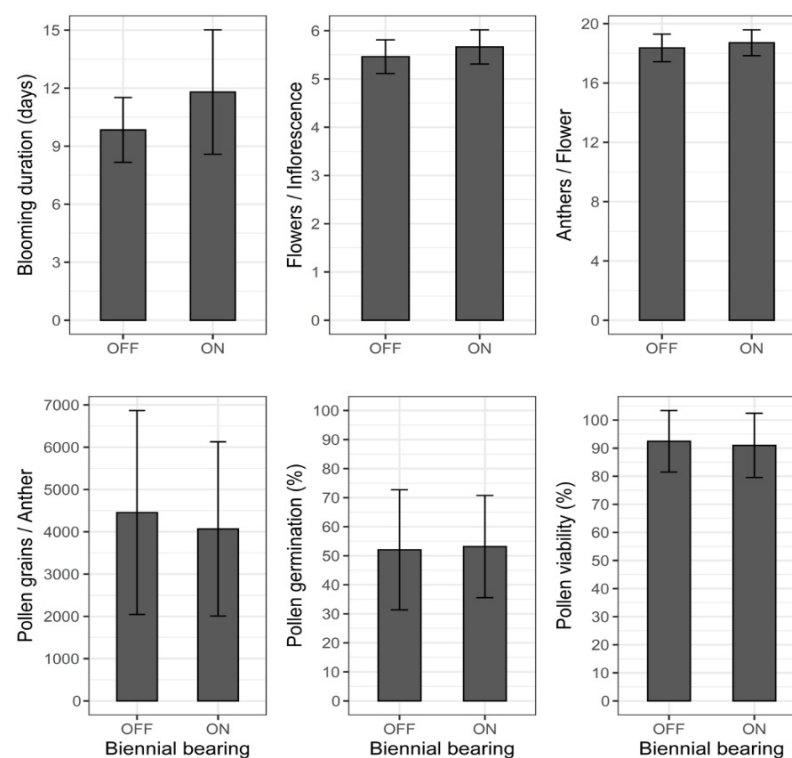


Figure 2. Effects of biennial bearing on floral and pollen biology attributes among a set of apple cultivars in Villaviciosa (north-western Spain). The biennial bearing effect is expressed as the difference in the mean values between the ‘on’ and ‘off’ years for six flowering-related traits. The vertical line shows the standard error of the mean.

3.4. Correlations between Floral and Pollen Traits

Among two successive seasons of phenotypic observations, Pearson's correlation coefficients indicated that most of the studied traits were poorly correlated to each other (Figure 3). A moderate to low positive correlation was observed between pollen germination and pollen grains per anther (Pearson's correlation coefficient; $r = 0.32$, $p = 0.01$). A negative correlation between flowering dates (day of the year) and the number of flowers per inflorescence was also found ($r = -0.36$, $p = 0.004$). A weak positive correlation was observed between bloom dates and pollen viability ($r = 0.3$, $p = 0.015$) and between the number of inflorescences/cm² of trunk cross-sectional area and the number of flowers per inflorescence ($r = 0.28$, $p = 0.021$). There were not significant correlations among the other variables at $p < 0.05$ (Figure 3).

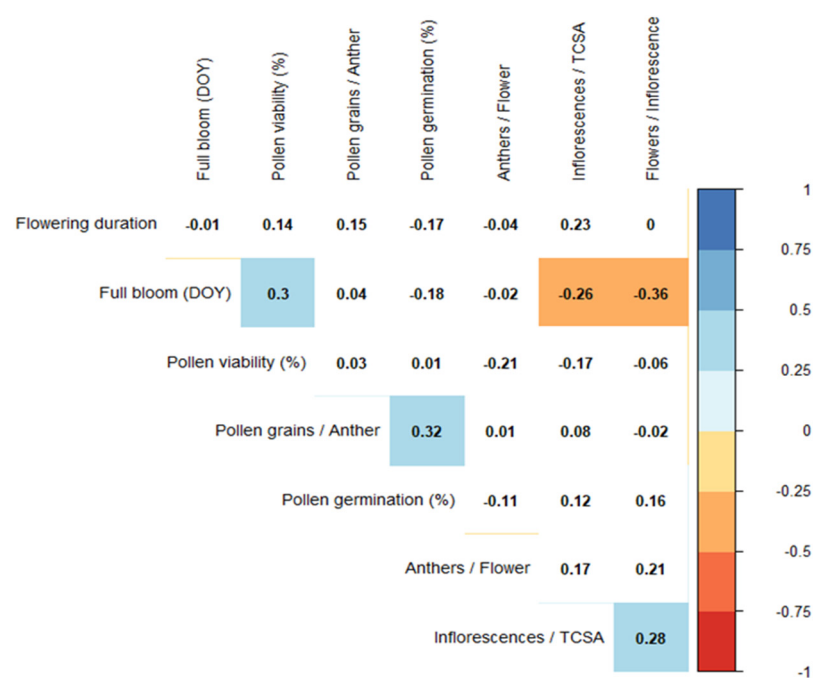


Figure 3. Pearson's correlation coefficients between floral and pollen characteristics used to characterize 45 apple cultivars in Villaviciosa (north-western Spain). The color gradient shows the strength of the correlation. Correlations that were not significant at $p < 0.05$ are represented by white boxes.

3.5. Evaluation of Apple Cultivars as Pollen Donors in Apple Orchards

Cultivars with medium-high biennial bearing tendency (less than 12 inflorescences/cm² of trunk cross-sectional area) were excluded, hence 14 cultivars were chosen as suitable pollinizers (Figure 4). According to this analysis, the cultivars 'X9406-11', 'Perurico' and 'Raxila Dulce' have the best attributes to be used as pollen-donating trees but other cultivars such as 'Raxona Ácida', 'Perurico Precoz', 'De la Riega' and 'Raxila Rayada' seem also to have great potential.

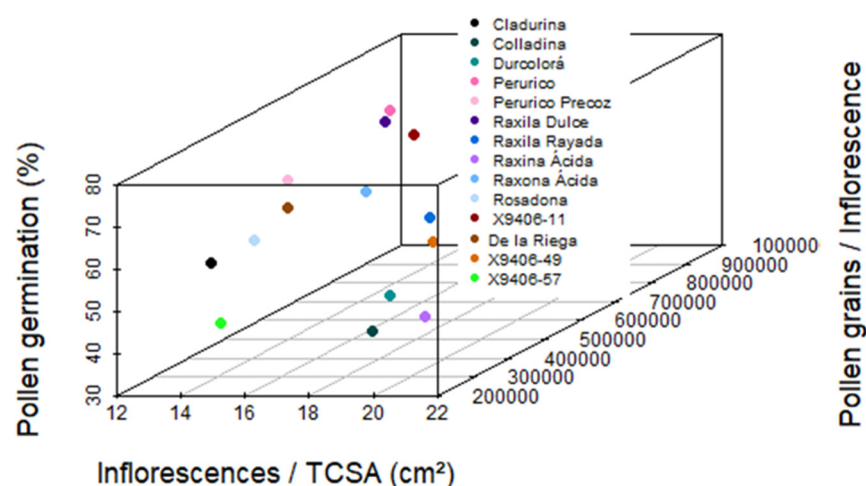


Figure 4. Three-dimensional scatterplot combining 3 variables (i.e., pollen germination (%), number of inflorescences/cm² of trunk cross-sectional area (TCSA) and number of pollen grains per inflorescence) for 14 apple cultivars in Villaviciosa (north-western Spain). The solid points stand for the means across the two-year data.

4. Discussion

4.1. Floral and Pollen Characteristics of Local Apple Cultivars

Blooming time and flowering duration for the same cultivar can vary considerably over years at the same location [37]. The duration of bloom is affected by temperatures and rainfall during this time [38]. Most of the previously published studies reported that the apple flowering period normally lasts between 10 and 17 days [39–41]. Across all cultivars and growing seasons, we found an average duration of 11 days, in line with a previous study in the same region by Dapena [26] who reported an average flowering time of 11.6 days across 104 Asturian cultivars. Flowering overlap is an essential prerequisite to ensure cross-pollination between trees in apple orchards [4,42]. According to our data, the possibilities of matching the flowering period of two or more cultivars are numerous owing to the broad range of flowering dates of the cultivars approved by the PDO appellation. Pollination can be more delicate in early flowering cultivars but some degree of bloom overlap between these cultivars occurred during both seasons. The only cultivar that might have had problems receiving pollen from other cultivars is the cv. ‘Raxao’. This very late-flowering cultivar flowered one week later than the immediately preceding cultivar. We hypothesized that this is a direct consequence of the very high chill and heat requirements exhibited by this cultivar [43] and the suboptimal winter chill conditions observed during these two seasons [44]. Apart from the coincidence in flowering dates, the duration of the bloom period is especially relevant since a long-lasting flowering period can lead to a more stable year-to-year fruit set. In general, the average duration of flowering seems adequate, although a quarter of the studied cultivars showed a two-year average shorter than 10 days in our region. These cultivars seem to have a relatively short period of pollen-receptive flowers and this situation might imply placing in the same orchard block other cultivars featuring optimal flowering-related characteristics to enhance cross-pollination. The cultivars ‘Raxona Ácida’ and ‘Raxona Dulce’ exhibited a long blooming duration of 16 days. These cultivars can potentially be used to increase flowering overlap or even to pollinate at the same time cultivars from different flowering groups. Minor differences between cultivars with respect to the number of flowers per inflorescence and the number of anthers per flower were found in our work. Regarding the number of anthers per flower, Church et al. [31] reported that dessert apple cultivars normally have a maximum of 20 anthers which is in agreement with our observations.

The analysis of pollen production has agronomic implications in fruit crops [45]. To achieve the best balance between pollen supply and demand in the orchard, it is important to choose cultivars which can guarantee a high production of viable pollen every year.

Additionally, the ability of the insects to pollinate effectively depends on the amount of pollen transported on their bodies and the number of pollen grains that they deposit on the stigmas [46,47]. Planting trees that produce a large quantity of high-quality pollen is expected to increase the pollen loads on insects, and this aspect has a direct effect on tree productivity and fruit quality [13,22]. Our results regarding the number of pollen grains per anther are, in general, consistent with previous reports since we found on average 4348 pollen grains per anther and 81,403 pollen grains per flower for the whole set of cultivars, but large variability among cultivars was observed. Dixon and Fuyon [48] found an average of 8428 pollen grains per anther in several apple cultivars. Other studies reported between 40,800 pollen grains per flower in cv. 'Jerseymac' [49] to 123,750 in the crab apple *Malus floribunda* (Javid et al., 2019). Pollen from fruit trees is often considered viable if the germination percentage value is >25% [50]. Among diploid cultivars, a germination percentage lower than 25% was only observed in cv. 'Fuentes' in 2018 and cultivars 'Prieta' and 'Raxina Ácida' in 2019, suggesting an adequate fertilization process for the large majority of cultivars. Moreover, our results regarding the pollen properties in apple trees support the ones described by Javid and Rather [51]. These authors found that the average pollen viability and pollen germination percentages across a set of varieties in India were 95.5% and 56%, respectively. Using a 10% sucrose medium, Albuquerque Junior et al. [52] observed pollen germination rates ranging between 59.6 and 73.2%, which are similar than the ones obtained in our work.

Although floral and pollen biology characteristics are variety-specific, some discrepancies with previous studies for the same variety are most likely the result of environmental and agronomic conditions [45,53,54]. Flower induction occurs in the year preceding bloom and floral and vegetative buds need to overcome a dormancy phase throughout the winter [55,56]. Since cultivar-specific chilling requirements for breaking dormancy must be fully satisfied [56,57], insufficient chill accumulation has a detrimental impact on the quality of blooming [58,59]. In Villaviciosa (north-western Spain), chill accumulation in seasons 2018/2019 and 2019/2020 was lower than the average chill accumulation from 1978 to 2019 [43] and it is possible that some cultivars did not reach their cultivar-specific chill requirement [44]. It is important to note that despite the high degree of phenotypic plasticity in response to temperature warming shown by some of these cultivars [43], large year-to-year fluctuations in flowering dates under our typical Oceanic climate conditions were observed. Because new plantings remain productive for many years, several cultivars will be advised for commercial orchards in north-western Spain to maximize flowering overlap and to avoid pollen limitations.

Since the start of the SERIDA apple breeding program, one of the main goals was to obtain regular-bearing cultivars. The sensitivity to alternate bearing of the Asturian local cultivars had been assessed on fruit yield [23,24]. The classifications based on yield can be sometimes misinterpreted as a result of multiple factors from peak of flowering until harvest time which can affect fruit production [60]. Most of the newly registered apple cultivars obtained by the SERIDA breeding program from crosses between local cultivars and other varieties showed a low tendency to alternate bearing based on flowering intensity data compared to the local germplasm.

4.2. Evaluation of Cultivar Suitability as Pollen Donors for the Local Apple Industry

Pollen yield per flower, flowering duration, pollen viability and return bloom are the main aspects explaining the potential of a particular cultivar as a pollen donor. Information regarding these flowering-related characteristics among a wide list of local cultivars and new varieties demonstrated that not all cultivars are adequate in terms of their pollinating potential. The results showed that several cultivars produced copious amounts of viable pollen in combination with an optimal level of return bloom without the implementation of thinning treatments. If blooming periods are similar, some of the best pollen-donating cultivars are the new cultivars 'X9406-11', 'Perurico' and 'Raxila Dulce'. In addition, 'Raxona Dulce' and 'Raxona Ácida' also feature good pollen properties and a particularly

long flowering period. *Malus floribunda* 821 met all the previously mentioned criteria but this species blooms very early in the study region, thus flowering synchronization with other cultivars is highly unlikely.

In Asturias, most of the current cider apple orchards are designed including several traditional cultivars [26]. In this work, we provide some insights regarding the best pollen donors for the local apple industry. Several cultivars meet the requirements to act specifically as pollinizers in the hypothetical case of the implementation of single-cultivar orchards. The presence of different cultivars presumably ensures pollen availability but also brings additional horticultural management challenges. Some of these challenges include specific pruning, plant protection and harvesting [61,62], as well as the choice of the adequate planting distance. However, considering the particularities of the local industry, we believe that the advantages of planting various cultivars outweigh the disadvantages. First, it has been reported that the presence of more than one variety in the same orchard helps to prevent the spread of diseases [63]. Secondly, the diversity of pollen can also increase the amount of pollen deposited on the stigmas and at the same time, increase the chance of receiving pollen from genetically compatible donors [64]. Another aspect to consider is that local cider industries normally prefer working with a combination of cultivars belonging to different technological groups according to their total acidity and phenolic content [23]. Finally, monovarietal orchards are highly sensitive to environmental conditions, thus orchards with multiple cultivars can minimize the negative impacts on productivity due to adverse weather conditions by diversifying the risks. Contrary to the classical approach of using exclusively pollinating varieties to supply pollen to the main variety, we found among our collection a number of cultivars showing at the same time excellent agronomic and fruit qualities and good performance as pollen donors. These pollinating cultivars have a long flowering period, low sensitivity to alternate bearing, produce a good supply of high-quality pollen and apples are also marketable for cider-making.

Further research should focus on pollen donor tree density and their distribution within the orchard, as well as the effect of different pollen sources on fruit yield and quality. In addition, more investigation is needed for new releases to determine cross-compatibility among cultivars. Some of the recently registered cultivars are genetically related so it can be expected that some of them do not cross-pollinate each other well (Delgado et al., in preparation). The characterization of the S-genotype of the traditional cultivars and the new releases can be particularly useful to increase the number of potential pollen donor cultivars for the dessert apple industry. Quinet et al. [65] stated that some old cultivars have rare S-genotypes, mainly those that were not used in breeding programs. Since most of the widely grown apple varieties are genetically related [66], the discovery of rare alleles among our collection may further increase the interest of some of these cultivars as pollinizers.

4.3. Correlations between Floral and Pollen Traits and Implications for Fruit Breeding Programs

Agronomic characterization in a wide range of phenotypic traits has been thoroughly carried out at SERIDA but the genetic variability among cultivars in terms of floral biology aspects had not previously been studied in detail. Breeding new apple cultivars requires a long time and involves many steps with the selection of the most suitable parents as the first step [67]. In the case of the studied apple cultivars, no obvious correlations were found between floral biology factors. Therefore, one parameter cannot be used to predict another one or, in other words, the change in one floral trait does not elicit statistically significant changes in other traits. Nonetheless, few weak statistically significant correlations were found among the studied traits. A positive correlation between pollen germination and pollen grains per anther may indicate that a higher production of pollen in a particular year positively affects the germination of pollen grains. A negative correlation between the flowering date (day of the year) and the number of flowers per inflorescence appears to be an indication of a lower number of flowers per inflorescence in late-flowering cultivars. A

positive correlation between bloom dates and pollen viability may suggest that presumably better environmental conditions prior to flowering in intermediate and late-blooming cultivars positively affect pollen development.

However, due to the great phenotypic diversity found in the apple collection, some specific characteristics can be useful for breeding purposes. For example, particularly relevant from the perspective of fruit breeding institutes is the level of return bloom. Some cultivars developed by the local breeding program such as 'Raxina Marelo', 'Raxila Rayada', 'Colladina' and 'Raxina Amarga' showed percentages of return bloom above 70%. Because the flowering pattern appears to be more cultivar-dependent than fruiting [68], this information can be used to breed regular-bearing cultivars. Likewise, the cultivar 'Durona de Tresali' bore a low number of flowers per inflorescence (4.2) in comparison with most of the cultivars which showed on average between 5.5 and 6 flowers. This genetic characteristic may potentially reduce the need for flower or fruit thinning. Furthermore, the identification of a broad range of flowering dates among the local and recently registered cultivars is expected to be helpful to design new crosses. Crossing some of these cultivars can lead to the development of new releases that flower during a specific period, providing some practical advantages, such as avoiding frost damage or facilitating cross-pollination with other cultivars.

On the other hand, there is scarce information related to the correlations of floral biology aspects in temperate fruit trees. Regarding the interaction between pollen quality and flowering dates, Ruiz et al. [69] found a significant negative correlation between pollen germination and flowering time in apricot varieties in south-eastern Spain, suggesting that late-flowering cultivars showed lower pollen quality than early-flowering cultivars in this species. On the contrary, we found that environmental conditions in our study site seem more favorable for late-flowering cultivars.

4.4. Floral Biology Traits and Quality of Pollen Grains Affected by 'On' and 'Off' Years

Significant differences between the 'on' and 'off' year were only found for the duration of flowering. Cultivars in an 'off' year showed a shorter duration of flowering than cultivars in an 'on' year. It seems reasonable to expect that a smaller number of inflorescences in an 'off' year can affect the length of the blooming in apple trees. Gallota et al. [70] found a significant difference for the number of pollen grains per anther between two successive years in apricot cultivars, although these authors observed that trees were able to produce the same amount of pollen grains per flower each year. In olive trees, Mazzeo et al. [28] and Methamem et al. [71] showed that trees produced fewer pollen grains but increased their values of pollen viability and germination during 'off' years to maximize ovule fertilization. Contrary to these results in olive groves, we predicted a similar efficiency in the rate of fertilization among a collection of apple cultivars. A significant trend toward both higher amount of pollen grains per anther and higher viability rates during an 'off' year was only observed for cv. 'Limón Montés'. This cultivar may have developed some adaptation mechanisms and its presence in the orchard may lead to a more stable production in the surrounding cultivars.

Nonetheless, further experimentation on the relationship between biennial bearing and flowering traits across a larger number of growing seasons, as well as a larger sample size may help to generate more conclusive findings regarding the aspects involved in the flowering behavior of this important fruit species.

5. Conclusions

Large phenotypic variability in floral biology traits was found in the set of apple cultivars even though most of the cultivars were selected in the study region and therefore have the same geographical origin. Our study covering two successive flowering seasons highlights some remarkable differences among apple cultivars in terms of flowering intensity and pollen biology attributes. Our estimations of pollen viability and germination seem sufficient for an optimal fertilization process but large differences among cultivars were

found regarding the production of pollen and the number of flowers per square centimeter of trunk cross-sectional area. Despite the floral biology parameters demonstrate genetic variability and thus breeding potential, our results also indicated that the characterization of floral and pollen characteristics appears to have limited applicability in apple breeding programs. The lack of obvious correlations between traits suggests that the studied traits need to be evaluated separately since traits cannot be used to predict each other. On the other hand, significant differences in pollen quality and quantity were not observed in the 'off' year with respect to the 'on' year in apple trees, suggesting a similar efficiency of the fertilization process during two successive flowering seasons. Finally, this work is expected to be useful for local growers in north-western Spain who want to decide the cultivar selection for new apple orchards. Cultivar mixtures will be advised for new plantings due to the large year-to-year fluctuations in flowering dates observed under an Oceanic climate.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11091717/s1>, Table S1: Reported parentage and flowering time (full bloom) of 45 apple genotypes in Villaviciosa (north-western Spain), Figure S1: Pollen production, pollen viability and pollen germination of apple pollen grains. (A) Apple pollen grains stained with aqueous eosin solution in a Malassez hemocytometer (10× magnification). (B) Pollen viability test of apple pollen grains using IKI stain (10× magnification). Pollen grains stained dark are scored as viable and unstained grains are counted as non-viable. (C) In Vitro pollen germination in concentrated agar (1.5%) containing 10% sucrose and boric acid (240 mg/L) after 24 hours at 21 °C. Pollen grains were considered germinated when the length of a pollen tube exceeded its diameter, Table S2: Percentage of inflorescences in an 'on' year which flowered again in an 'off' year among a list of 24 apple cultivars, Figure S2: Flowering period of 44 apple (*Malus domestica* Borkh.) cultivars in two successive flowering seasons (i.e., 2018–2019 or 2019–2020) in Villaviciosa (north-western Spain). F1, F2 and G stand for the date of first bloom, full bloom and petal fall, respectively. The solid colour bars indicate the blooming duration for each flowering season. Symbol "*" stands for missing phenological data.

Author Contributions: A.D. designed the experiment with the assistance of E.D. and M.Q. A.D. recorded the data and analyzed the results. A.D. wrote the manuscript and all the authors contributed to analyzing and discussing the results. E.D. acquired the funding. All authors have read and agreed to the published version of the manuscript.

Funding: Funding was provided by an FPI-INIA fellowship to AD (CPD-2016-0190), MINECO-INIA (FEDER. UE) and MICIU-INIA-AEI through project RFP2015-00022, RTA2014-00 and RTA2017-00102-C03-01.

Data Availability Statement: Data available on request due to restrictions eg privacy or ethical.

Acknowledgments: The authors are grateful to Anne-Laure Jacquemart for hosting AD at Université Catholique de Louvain. We would like to thank Héctor García for his technical support during the second experimental season.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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