

1 **Anther meiosis time is related to winter cold temperatures in apricot (*Prunus***
2 ***armeniaca* L.)**

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1 **ABSTRACT**

2 In temperate woody plants, flowering time is adjusted to the environmental temperature, and
3 survival to cold winter temperatures is achieved through dormancy. But also chilling
4 temperatures during dormancy are required for proper flower bud development and flowering.
5 The time the flower bud remains dormant is both genetically and environmentally controlled,
6 and is a major factor determining the adaptation of species and cultivars to particular
7 ecological conditions because chilling requirements vary greatly among genotypes. Recently
8 this adaptation is jeopardised with climate changing conditions. However, little is known on
9 the biological milestones underpinning these events, and when differences in flowering time
10 are established. In this work we evaluate the hypothesis that anther meiosis time is related to
11 winter cold temperatures, and that differences in flowering time are set up by differences in
12 the time when pollen meiosis occurs. For this purpose, anther development has been
13 characterized in five apricot cultivars with different chilling requirements. The work was done
14 over two years with different weather conditions, a cold and a mild winter. The sporogenous
15 tissues differentiated prior to dormancy and remained in this apparently quiescent stage
16 during the winter. Once chilling requirements were fulfilled, meiosis closely followed and
17 was highly correlated to breaking of endodormancy. Meiosis was completed within one week
18 and was followed by a change in the colour of the anthers from green to yellowish, which
19 could be a useful visual indicator to know that breaking of endodormancy had already
20 occurred. The fact that this sequence of events was consistent in all the cultivars, and different
21 climatic years analyzed, supports the hypothesis that winter cold temperatures are related to
22 the time of pollen meiosis, which in turn reflects in different flowering times.

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24 *Key-words: Prunus armeniaca; chilling temperatures; anther development; pollen; meiosis;*
25 *endodormancy.*

26

1 **1. Introduction**

2

3 Perennial plant acclimation to different latitudes requires the adaptation to cold winter
4 conditions. Woody plants enter dormancy during the winter and resume growth prior to
5 flowering the next spring (Perry, 1971). During dormancy, meristems are unable to initiate
6 growth even under favourable environmental conditions (Cooke et al., 2012; Rohde and
7 Bhalerao, 2007). The time that the flower buds remain dormant is genetically and also
8 environmentally controlled (Horvath *et al.*, 2003). Whereas entering dormancy is
9 accompanied by internal changes that prepare buds to withstand freezing temperatures (Gusta
10 and Wisniewski, 2013; Welling and Palva, 2006), a number of changes have been reported
11 during and at the end of dormancy as hormones concentration, lipids and proteins profiles,
12 and water and sugar content (Arora et al., 2003; Horvath et al., 2003; Kalberer et al., 2006).
13 But little is known on the biological events underpinning breaking of dormancy, apart that it
14 is followed by bud burst, although often a good lapse of time occurs between these two
15 events, and a biological indicator is so far missing and elusive.

16 To know the biological milestones of this process would provide a frame to
17 understand chilling requirements and their implications. In temperate fruit trees species,
18 chilling is required for proper flower bud development and flowering after emerging from
19 winter dormancy (Perry, 1971). Chilling requirements are highly variable among species and
20 cultivars, and have a clear agronomic and economic reflection because they determine the
21 adaptation of each cultivar to particular areas of fruit production, since each genotype has
22 particular cold requirements, which are constant over years and sites (Bohlenius *et al.*, 2006).
23 Also this is the main restriction for the cultivation of temperate fruit trees to warmer latitudes
24 (Horvath *et al.*, 2003). Furthermore, global warming is jeopardising plant reproduction
25 (Hedhly et al., 2009), and some temperate fruit trees are potentially at risk in traditional fruit

1 growing regions due to the lack of winter chilling caused by climate change (Atkinson et al.,
2 2013; Campoy et al., 2011; Luedeling et al., 2011). Whereas variations in chilling
3 requirements among genotypes are of great economic importance, little is known on the
4 biological events underlying these differences (Faust et al., 1997; Jones et al., 2013; Rohde
5 and Bhalerao, 2007) and their influence in flower bud dormancy (Bartolini et al., 2006;
6 Weinbaum et al., 1989). Depending on the influence of physiological or environmental
7 factors, dormancy during winter has been classified as endodormancy (regulated by internal
8 physiological factors) and ecodormancy (regulated by environmental factors) (Lang *et al.*,
9 1987). From an agronomic point of view, endodormancy in temperate fruit trees ends when
10 the chilling requirements are fulfilled. After that, bud growth is inhibited by unfavourable
11 environmental conditions, mainly cold, during ecodormancy. But it is difficult to establish
12 when flower buds have broken endodormancy and entered ecodormancy, since no external
13 signs of development can be detected in either of these phases, until the end of ecodormancy
14 that takes place several weeks later at budburst, when the bud scales separate and the bud
15 swells (Julian et al., 2010).

16 Several models have been proposed to estimate the chilling requirements during
17 dormancy of particular cultivars mainly out of statistical correlations between flowering dates
18 and seasonal temperatures in the field (Alonso et al., 2005; Bidabé, 1965; Cesaraccio et al.,
19 2004; Dennis, 2003; Luedeling and Gassner, 2012; Luedeling et al., 2009). Alternatively,
20 breaking of endodormancy has been estimated by cutting shoots from dormant trees at various
21 times during the winter, exposing them to warm forcing conditions, and quantifying chilling
22 temperatures up to cutting time when a percentage of flower buds is able to increase in weight
23 (Brown and Kotob, 1957) or show external phenological development (Weinberger, 1950).
24 These approaches have been used over half a century to calculate chilling requirements in a
25 number of cultivars of different fruit tree species. In spite of the empiric nature of these

1 models, they are useful to determine if a particular genotype can adapt successfully to a new
2 area or for genetic selection of parental lines in breeding programs (Albuquerque et al., 2008;
3 Alonso et al., 2005; Egea et al., 2003; Gao et al., 2012; Jones et al., 2013; Ruiz et al., 2007;
4 Vahdati et al., 2012). However, chilling requirements have not been estimated in many
5 cultivars, and when they are established, results are highly variable mainly by the fact that
6 these models are site- and species-specific (Cesaraccio et al., 2004; Luedeling, 2012), by the
7 absence of unity in the timing of the start and the endpoint when temperature records are
8 considered (Dennis, 2003) and by the differences among studies concerning the forcing
9 conditions, the percentage of weight increase and the phenological stage of bud development
10 considered as indicators of breaking dormancy. These problems are difficult to overcome
11 because there is a lack of knowledge on the physiological events underpinning entering and
12 breaking endodormancy (Cesaraccio et al., 2004; Fuchigami and Wisniewski, 1997; Lang et
13 al., 1987; Luedeling, 2012), which prevent to take into account tree physiology to establish
14 the period in which to quantify chilling.

15 Recent work in apricot (*Prunus armeniaca* L.) showed that stamen development is a
16 valuable tool to frame dormancy. Dormancy mark a boundary between the development of
17 the sporogenous tissue in the anther and the occurrence of pollen meiosis (Julian *et al.*, 2011).
18 While these results were neat for one cultivar, the question remains on whether this occurs in
19 different cultivars and weather conditions. Whenever pollen meiosis has been examined in
20 woody plants, it has been reported to occur around breaking of dormancy, either before
21 (Bartolini et al., 2006; Jedrzejuk and Szlachetka, 2005; Owens and Molder, 1971; Zhang et
22 al., 2007), during (Bartolini et al., 2006; Weinbaum et al., 1989) or after breaking dormancy
23 (Gifford and Foster, 1987; Luomajoki, 1982; Sedgley and Griffin, 1989). While these reports
24 are clearly controversial, they share a common ground, and this is that in a number of
25 unrelated species from gymnosperms to angiosperms, meiosis appears to occur around

1 breaking of endodormancy. This variation may be due to differences in the timing of pollen
2 meiosis among species or alternatively, and more likely, in the different ways to determine the
3 breaking of endodormancy from empirical models.

4 In apricot, each cultivar is usually restricted to a particular geographical area with
5 certain ecological conditions, and low yields are obtained whenever particular cultivars are
6 grown in other areas (Layne et al., 1996; Ledbetter, 2008). This narrow adaptability limits the
7 expansion of most apricot cultivars to other regions and countries. However, the causes of this
8 low adaptability are not understood (Hormaza *et al.*, 2007).

9 In this work we evaluate the hypothesis that winter cold temperatures affect the time
10 of pollen meiosis, which in turn reflects in differences in flowering time. For this purpose,
11 anther development was examined in several apricot cultivars with different chilling
12 requirements, analyzing the relationship between pollen meiosis and chilling fulfillment. The
13 work was performed over two years with different weather conditions, one with a cold and the
14 other with a warm winter.

15

1 **2. Material and methods**

2

3 *2.1. Plant material*

4

5 Trees of five apricot cultivars ('Canino', 'Corbato', 'Moniqui', 'Paviot' and 'Luizet')
6 with different chilling requirements were selected from an experimental orchard located at
7 CITA in Zaragoza (Spain) at 41°44'30"N, 0°47'00"W and 220 m altitude. The experiment was
8 performed over two years with different weather conditions: a cold and a mild winter.

9

10 *2.2. Quantification of chilling*

11

12 The dates of chilling fulfilment of each cultivar were estimated as the number of hours
13 below 7.2 °C (chilling hours, CH) (Weinberger, 1950), according to the chilling requirements
14 previously calculated for these cultivars in the same conditions of the experiments here
15 reported (Tabuenca, 1968): 'Canino', 711-779; 'Corbato', 750-846; 'Moniqui', 779-926;
16 'Paviot', 995-1075; 'Luizet', 1058-1116. Daily records of temperature were registered in a
17 meteorological station located at the research centre. The date of chilling fulfilment for each
18 cultivar and year was related to developmental events observed in the flower buds.

19

20 *2.3. Flower bud development*

21

22 In order to follow internal and external flower bud development, 15 flower buds were
23 weekly sampled from December to February over the two years of experiments. To determine
24 the flower bud stage and the colour of the anthers, five flower buds per cultivar and sample
25 date were dissected with the help of an ophthalmologic scalpel and observed under a

1 stereoscopic microscope (Leica MZ-16, Cambridge, UK). The colour of anthers was assessed
2 from dormancy to bloom using a RHS Colour Chart (Royal Horticultural Society, London,
3 England). External phenological stages of flower bud from dormancy to bloom were
4 characterized according to Baggiolini (1952).

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6 *2.4. Microscope preparations*

7

8 To examine the internal development, 10 flower buds per cultivar and sample date
9 were weighted and fixed in ethanol:acetic 3:1 (v/v). Anthers were removed from fixed buds
10 with the help of a scalpel and mounted by squash with a solution of 0.25 µg/ml of DAPI in
11 0.05 M in TRIS buffer (pH 7.2) for 1 h at room temperature in a light-free environment to
12 observe nuclei (Williams *et al.*, 1999), followed by adding 0.1% aniline blue in 0.1N K₃PO₄
13 (Currier, 1957) to stain also callose.

14 Five additional flower buds per sample date were fixed in glutaraldehyde at 2.5% in
15 0.03 M phosphate buffer (Sabatini *et al.*, 1963), dehydrated in an ethanol series and embedded
16 in JB4 plastic resin (Polyscience Inc., Warrington, Philadelphia, PA, USA), sectioned at 2 µm
17 in a multicut microtome (Leica 2045, Cambridge, UK) and stained with 0.07 % calcofluor in
18 water for cellulose (Hughes and McCully, 1975), or with 0.01% auramine in water (w/v) for
19 cutin (Heslop-Harrison, 1977).

20 Microscopic preparations were observed under an Olympus BH2 microscope
21 (Olympus Optical Co, LTD, Japan) with UV-epifluorescence using a BP-405 exciter filter and
22 a Y-455 barrier filter.

23

24 *2.5. Statistical analyses*

1 Dates of chilling fulfilment, pollen meiosis, change of anther colour from green to
2 yellowish and full bloom were determined in the five apricot cultivars over two years.
3 Correlations between the dates were analysed by Pearson correlation coefficients performed
4 using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA).
5

1 **3. Results**

2

3 The theoretical chilling requirements for each apricot cultivar were fulfilled 10-15
4 days before in the cold than in the mild winter (Fig. 1). Taking together the five cultivars over
5 the two years, the fulfilment of the different chilling requirements elapsed over one month. In
6 the cold winter year, chilling requirements were fulfilled before, but the subsequent flower
7 bud development was also slower than in the mild winter year, resulting in similar flowering
8 times (Fig. 2). But the period of time between chilling fulfilment and budburst was variable
9 depending on cultivars and years, especially in the mild winter (Fig. 2).

10 Anther development was first observed in sections. Callose layering around the pollen
11 mother cell was the first clear sign of the onset of meiosis (Supplemental Fig. 1a). Meiosis
12 proceeded rapidly and was completed within one week between the first callose layering
13 around the pollen mother cell and the transition from tetrads (Supplemental Fig. 1b) to young
14 microspores (Supplemental Fig. 1c). For a rapid screening of meiosis in relation to dormancy,
15 squash preparations were evaluated. Over the two years, sporogenous tissue (Supplemental
16 Fig. 1d) was observed during endodormancy in all the cultivars analyzed. Callose layering
17 around the pollen mother cells occurred concomitantly with the initiation of prophase I
18 (Supplemental Fig. 1e) and continued on subsequent meiotic phases: Telophase I
19 (Supplemental Fig. 1f), Prophase II (Supplemental Fig. 1g), Anaphase II (Supplemental Fig.
20 1h) and Telophase II (Supplemental Fig. 1i). Then tetrads were apparent (Supplemental Fig.
21 1b), closely followed by the release of the young microspores (Supplemental Fig. 1c).

22 Although the onset of pollen meiosis was variable between cultivars and years, the
23 steps of anther development from endodormancy to budburst were conserved in an orderly
24 way in both years (Fig. 2). Following the same pattern of bud development, differences
25 between cultivars in anther development (Fig. 2) were more conspicuous in the mild winter

1 year. But in both years, differences in timing were observed between the cultivars examined
2 according to their chilling requirements. The onset of male meiosis was detected in each
3 cultivar and year at different times, and this time always occurred once the particular chilling
4 requirements were fulfilled, but with flower buds still in stage A, closed and without external
5 signs of development. Thus, those cultivars that underwent first male meiosis flowered earlier
6 than those that reached meiosis later, and those cultivars with low chilling requirements
7 underwent meiosis earlier than those with high chilling requirements (Fig. 2).

8 Observations from dissected flower buds between endodormancy and early budburst
9 did not show any signs of internal development during endodormancy, when the colour of
10 anthers were light-green (RHS codes YG-144C and, YG-N144D), corresponding to
11 sporogenous tissue (Supplemental Fig. 2a). However, the colour of anthers changed from
12 light-green to green-yellowish (RHS codes YG-N144A, YG-151D, YG-151A) just following
13 meiosis (Supplemental Fig. 2b). At the young microspore stage, the anthers became yellowish
14 (RHS codes GY-1B, Y-1A) (Supplemental Fig. 2c), concomitantly with the intense phase of
15 exine development in the young microspores. Finally, when flower buds initiated budburst,
16 the anthers presented an intense yellow colour (RHS codes Y-2B, Y-2A, Y-3B)
17 (Supplemental Fig. 2d) and microgametogenesis started with the first mitotic division.

18 The comparison of five cultivars with different chilling requirements over two years
19 with a cold and a mild winter showed that although differences in timing were observed
20 between cultivars and years, in all cases the same sequence of events followed breaking of
21 endodormancy. After the fulfilment of chilling requirements, pollen meiosis occurred and was
22 followed by the change in the colour of anthers, which was concomitant with an increase in
23 bud weight (Fig. 3). In the different cultivars and years, the time lapse from the fulfilment of
24 chilling requirements to bud burst ranged from two to six weeks. But the fulfilment of chilling
25 requirements and meiosis took from two to three weeks.

1 Taking the five cultivars over the two years showed that while no or very weak
2 correlation could be found between chilling fulfilment and bud burst, or full bloom, a positive
3 significant correlation could be found between chilling fulfilment and meiosis or anther
4 colour; and these two parameters were also correlated with full bloom (Table 1).
5

1 4. Discussion

2

3 The data from this initial study indicate that winter cold temperatures clearly have a bearing
4 on the time of pollen meiosis, which in turn is reflected in flowering time. Endodormancy
5 break is followed by a conserved sequence of events; pollen meiosis is one of the first
6 indicators, which is closely followed by a change in the anther colour that can be seen to the
7 naked eye.

8

9 4.1. Meiosis and chilling accumulation

10

11 While differences in the time when meiosis started were found between cultivars and
12 years, pollen meiosis took place once chilling requirements had been fulfilled in all
13 circumstances. Meiosis was completed in around one week, a period of time considerably
14 longer than in other species (Bennett, 1977), but shorter than in other woody species
15 (Luomajoki, 1982). The date of microspore meiosis is highly variable among *Prunus* species
16 (Julian et al., 2011; Kadir and Proebsting, 1994; Ontivero et al., 2005; Soodan et al., 1988).
17 However, when meiosis has been analysed among cultivars of the same *Prunus* species,
18 results are not always clear; and meiosis has been reported to occur concomitantly in apricot
19 cultivars with different chilling requirements (Bartolini *et al.*, 2006) and in different peach
20 and almond cultivars (Soodan *et al.*, 1988). Conversely, differences in the time of pollen
21 meiosis have been reported in peach cultivars (Citadin et al., 2002). Results herein showed
22 differences between apricot cultivars and years, but these differences were highly correlated
23 with the fulfilment of chilling requirements.

24 Pollen meiosis and its genetic control are known to be highly conserved processes in
25 plants (Dickinson and Grant-Downton, 2009; Pacini, 2010; Scott et al., 2004; Wilson and

1 Zhang, 2009), but the causes behind the triggering of the process remain elusive (Bennett,
2 1977; Bhatt et al., 2001; Harrison et al., 2010; Wilson and Yang, 2004). Pollen meiosis is
3 influenced by cold stress (Thakur et al., 2010), and has also been related to previous heat
4 requirements, either in sub-tropical climates (Citadin et al., 2002) or under forcing conditions
5 (Whelan et al., 1968). Differences here reported between cultivars support its genetic control,
6 but clear differences between the years here observed confirm the environmental regulation of
7 the process.

8

9 *4.2. Change of anther colour*

10

11 After the completion of meiosis, pollen exine quickly developed (Julian et al., 2011) as
12 it has also been reported in other species (Blackmore et al., 2007; Lora et al., 2009). This was
13 reflected in that the anthers acquired a distinctive yellowish colour that could be observed by
14 the naked eye. This visual indicator announced that the flower bud was no longer dormant.
15 The fact that the date in which anthers changed in colour from green to yellowish varied
16 between cultivars and years, and was positively correlated to the dates of pollen meiosis, and
17 chilling fulfilment further validates the change in anther colour as a good external marker
18 showing that the flower bud is no longer dormant.

19 In spite of the high correlations obtained, neither the change in anther colour, nor
20 meiosis, were concomitant with the fulfilment of chilling requirements, and thus could not be
21 used to evaluate chilling requirements, as it occurs in subtropical conditions (Citadin et al.,
22 2002). But both are a much more precise indicator, than the so far used bud burst, reporting
23 that flower buds have already broken dormancy. Also the time elapsing between chilling
24 fulfilment and meiosis sets a neat frame where to search for events accompanying breaking of
25 endodormancy.

1 Further work is required to evaluate whether the changes reported here in meiosis and
2 change in anther colour could also be used to report on the breaking of endodormancy in other
3 species. But the fact that pollen development is a highly conserved process in plants (Julian et
4 al., 2011; Sanders et al., 1999), together to the simplicity of the tests here reported opens the
5 way for this evaluation.

6

7 *4.3. Conclusion*

8

9 Overall, in all the apricot cultivars observed herein and over both years pollen meiosis
10 closely followed the fulfilments of chilling requirements. Moreover this was followed by a
11 change in anther colour that was visible to the naked eye. The good correlations found
12 between chilling requirements, meiosis and flowering time shows that winter cold conditions
13 have a clear bearing on the time of meiosis, which in turn is highly correlated to flowering
14 time. While meiosis and anther colour cannot be used as a measurement of chilling
15 requirements, they are clear reporters showing that flower buds have broken dormancy. We
16 hope that this work will result in a more comprehensive understanding of dormancy in woody
17 perennials and will contribute to the development of new methods to calculate chilling
18 requirements.

19

20 **Acknowledgments**

21 We thank Rosa Fustero and Reyes López for technical assistance. Financial support
22 for this research was provided by Ministerio de Economía y Competitividad - European
23 Regional Development Fund, European Union (AGL2009-12621-C02, AGL2012-40239) and
24 Gobierno de Aragón - European Social Fund, European Union (Grupo de Investigación de

1 Aragón A-43). C. J. was supported by a fellowship of INIA (Instituto Nacional de
2 Investigación y Tecnología Agroalimentaria).

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15

1 **Figure captions**

2

3 **Fig. 1.** Chilling accumulation and chilling fulfilment (arrows) of five apricot cultivars in two
4 years with a cold and a mild winter.

5

6 **Fig. 2.** Anther development from dormancy to flowering in five apricot cultivars in two years
7 with a cold and a mild winter. ✿: Chilling fulfilment, C: Callose layering around pollen
8 mother cells, T: Tetrads. Dashed lines delineate the flower bud stages of development
9 according to Baggiolini (1952). A: Closed flower bud without external signs of development.
10 B: Separation of bud scales. C: Exposed red sepals. D: Exposed petals. F: Anthesis.

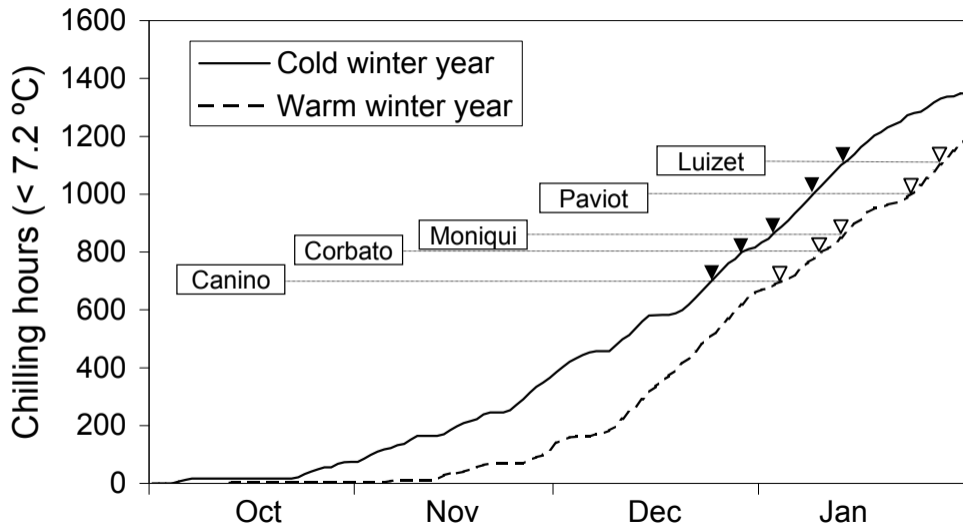
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12 **Fig. 3.** Flower bud growth from dormancy to bud burst in five apricot cultivars, showing the
13 dates of chilling fulfilment, meiosis and change of anther colour from green to yellowish over
14 two years with a cold (black symbols) and a mild winter (white symbols).

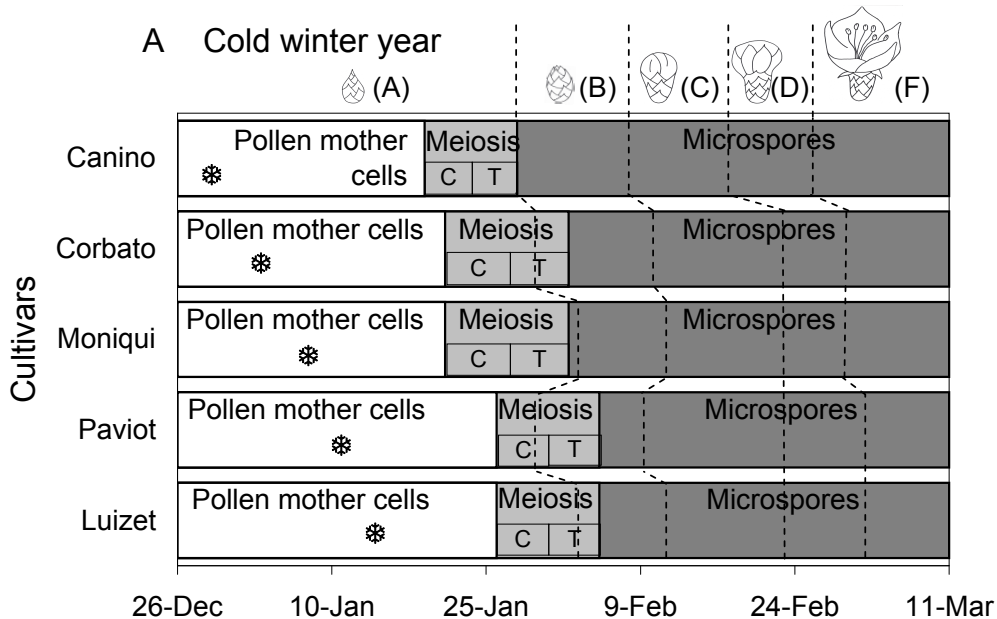
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16 **Supplemental Fig. 1.** Microsporogenesis in apricot. (A) Pollen mother cells surrounded by
17 callose layering during male meiosis, (B) tetrads and (C) young microspores with a
18 conspicuous exine. (D) Sporogenous tissue. (E) Prophase I (diakinesis) and Metaphase I. (F)
19 Telophase I. (G) Prophase II. (H) Anaphase II. (I) Telophase II. Transversal 2 μ sections of
20 anthers embedded in JB4 and stained with calcofluor (A, B) and with calcofluor and auramine
21 (C) Squashed anthers double stained with aniline blue and DAPI (D, E, F, G, H, I). Scale
22 bars: 20 μ m.

23 **Supplemental Fig. 2.** Anther colour from dormancy to budburst changed from (A) light-
24 green during endodormancy at stage A, (B) to green-yellowish in ecodormancy at the same
25 phenological stage A, (C) to yellowish at stage B, and (D) to intense yellow colour at stage C.



A Cold winter year



B Warm winter year

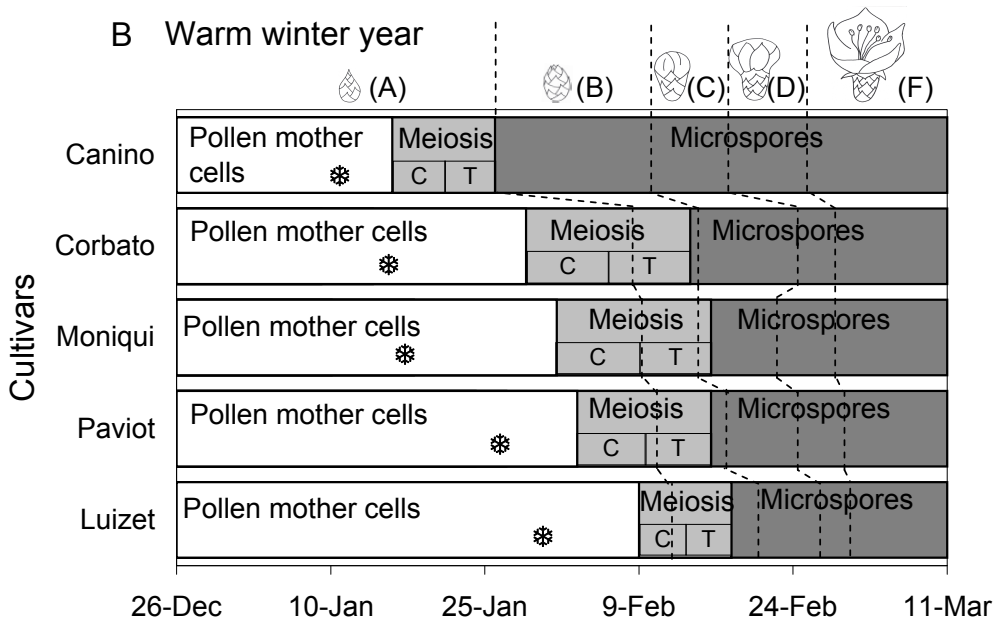


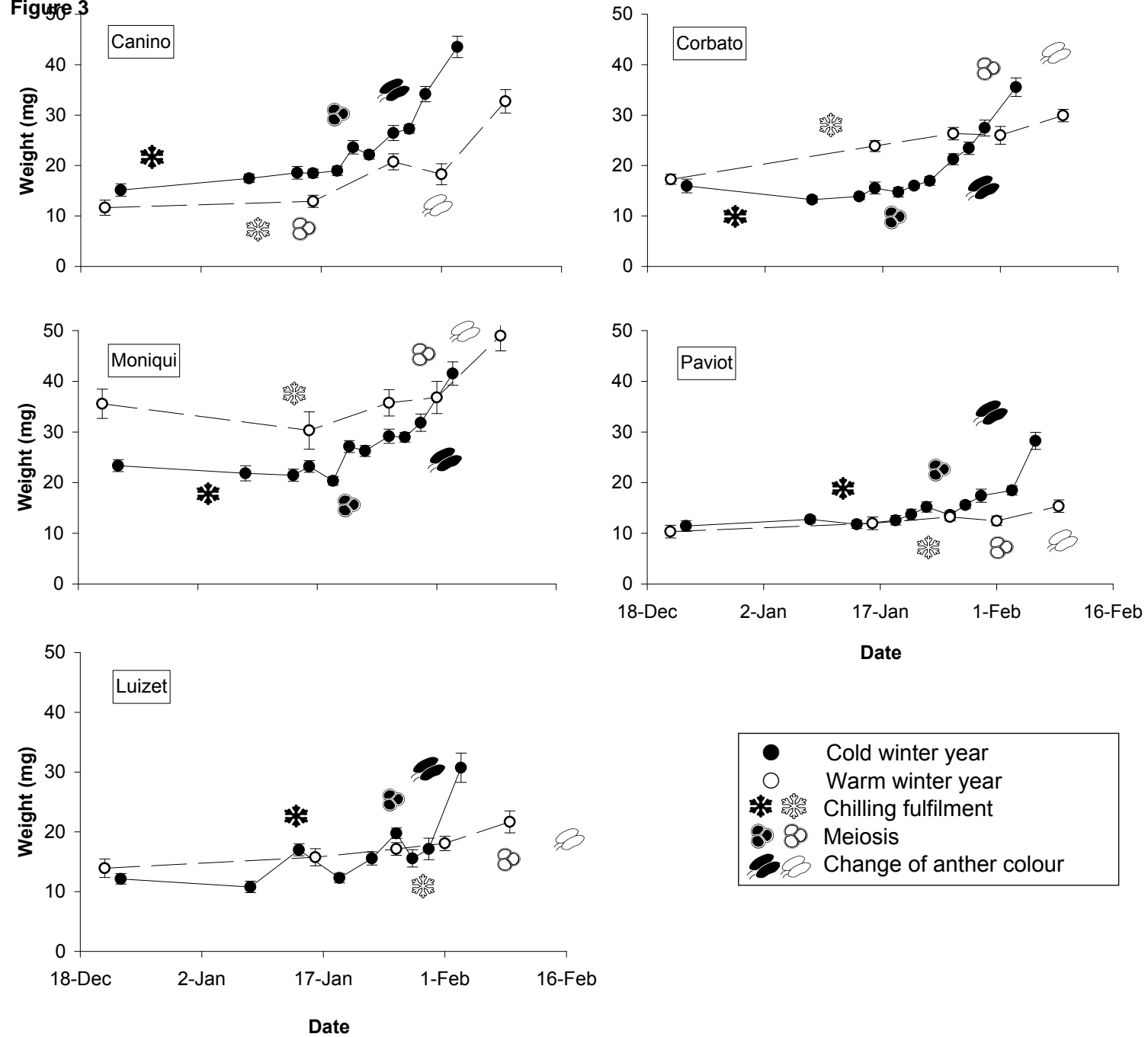
Figure 3

Table 1. Pearson's correlation coefficients between the dates of chilling fulfilment, budburst, pollen meiosis, change of anther colour from green to yellowish and full bloom in 5 apricot cultivars in two years with a cold and a mild winter.

	Cold winter year	Warm winter year
Budburst / Chilling fulfilment	0.593 ^{NS}	0.828 ^{NS}
Budburst / Full bloom	0.609 ^{NS}	0.993 ^{**}
Pollen meiosis / Chilling fulfilment	0.978 ^{**}	0.892 [*]
Pollen meiosis / Anther yellowish	0.968 ^{**}	0.975 ^{**}
Pollen meiosis / Full bloom	0.890 [*]	0.988 ^{**}
Anther yellowish / Chilling fulfilment	0.969 ^{**}	0.911 [*]
Full bloom / Chilling fulfilment	0.911 [*]	0.863 ^{NS}
Anther yellowish / Full bloom	0.976 ^{**}	0.961 ^{**}

* $P < 0.05$; ** $P < 0.01$; NS Not significant.

