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Flower bud development of almond cultivars based on three different methods

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ABSTRACT

Flower bud development of fruit trees plays a key role in their climatic adaptation. It is closely related to dormancy release that determines winter frost susceptibility. Detailed characterisation of flower bud development of 25 almond (*Prunus amygdalus* L. Batsch) accessions representing wide range of flowering times have been performed by microsporogenesis and pistil growth studies for 3 years. Six developmental stages were distinguished in the process of microsporogenesis, while pistil development could be classified into four phases. The examined cultivars showed significant differences in the length and occurrence of microspore developmental stages and year effect was observed. On the basis of the length of microsporogenesis stages, cultivars were clustered into five main groups. The shortest periods of archesporium and microsporogenesis as a sum were detected in accessions 'Eriane', '5/15' and '1/7' (with an average of 20 and 138 days in all three), while the longest ones were determined in 'Constanti' and 'Vairo' (65 and 160 days in both), respectively. The increment of pistil length was suspended during the dormancy period and after dormancy release, it was accelerated first at a slow rate followed by a few days of rapid growth prior to blooming. In order to determine the date of endodormancy release, these three methods – microsporogenesis, pistil length studies, and forcing of shoots – were analysed. All methods revealed significant differences among accessions. The dormancy release estimated by microsporogenesis studies showed the highest variability among the three methods used.

Keywords: endodormancy, forcing, microsporogenesis, *Prunus amygdalus,* pistil length

INTRODUCTION

In most temperate fruit trees, flowering occurs in spring but flower bud differentiation starts the previous summer. Flower buds enter dormancy during the winter and resume their growth prior to flowering (Julian et al., 2009). Flower bud development slows down during endodormancy, during which period, there is no visible change and it accelerates again until bloom (Szalay, 2006). Flower bud developmental process is determined genetically (Lamp et al., 2001; Szalay, 2006), but the process is influenced to a great extent by environmental factors (Szalay, 2006). Chill accumulation during the dormancy period allows the gradual change from flower bud endodormancy to flower bud ecodormancy where flower bud development

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is controlled by subsequent heat accumulation (Egea et al., 2003; Bartolini et al., 2006a; Hajnal et al., 2013; Sanchez-Perez et al., 2014; Szalay et al., 2019). In temperate climates, cultivars with fast flower bud development are highly exposed to the danger of frost. Cultivars having fast flower bud development have short endodormancy period, therefore winter frosts can occur. Moreover, their blooming starts early and the flowers are more exposed to spring frost (Szalay and Németh, 2010; Hajnal et al., 2013). Early flowering almonds are a good choice for growing in warm areas where there are problems of insufficient chilling (Prudencio et al., 2018). Flower bud development of other *Prunus* species such as apricot and sweet cherry has been studied (Szalay and Németh, 2010; Fadón et al., 2019), however, no such detailed description is available on flower bud development of almonds.

Flower bud development can be carefully studied during the dormancy period by microsporogenesis methods. The microsporogenesis development of apricot flower buds was studied during winter (Szalay, 2006; Szalay and Németh, 2010; Németh, 2012; Hajnal et al., 2013; Szalay et al., 2019). Six stages of microsporogenesis were identified and the time to each stage of development varied depending on genotypes. In addition, in apricot cultivars (Viti and Monteleone, 1991), variability among cultivars was observed in the formation of the tetrad stage of microsporogenesis. However, in the case of the formation of young pollen grains, the authors observed less variability among the cultivars. The relationship between the end of dormancy and microsporogenesis in apricot flower buds was studied; the meiosis was indicated as a promising biomarker for the determination of dormancy release (Bartolini et al., 2006a, 2006b; Herrera et al., 2022). Changes in frost tolerance in relation to the developmental rate of floral buds were determined by studying their microsporogenesis (Szalay et al., 2006). These authors reported that apricot cultivars with short endodormancy or quick floral bud development had the weakest winter hardiness. Knowledge of a given cultivar's chilling and heat requirements is important as it determines its adaptation to specific ecological conditions (Bassi et al., 2006; Julian et al., 2009). These agronomic requirements for specific cultivars can be accurately estimated by determining the endodormancy release. Different methods have been used to determine dormancy release. However, knowledge of phenological and biological markers linked to dormant conditions remains scarce, making it difficult to establish the end of endodormancy (Herrera et al., 2022). There has been no comparison study of the different methods of establishing dormancy release in almonds so far. Dormancy release has been determined by both statistical correlation (Alonso et al., 2005) and partial least squares (PLS) regression (Luedeling et al., 2013). Such a correlation was not observed regarding cultivars with a longer endodormancy period. The results of both types of approaches are not directly comparable under different conditions (Fadón et al., 2018). Herrera et al. (2022) compared three methods for indirectly estimating dormancy release by forcing shoots and two statistical approaches that related seasonal temperatures and blooming dates. All three methods estimated different dates for endodormancy end. Male meiosis was indicated as a possible biomarker to determine dormancy release and estimate apricot agroclimatic requirements. The relationship between dormancy and microsporogenesis in apricot flower buds was studied by Hajnal et al. (2013); Julian et al. (2009); Szalay et al. (2019). The differentiation of archesporium tissues or beginning meiosis was indicated as a biomarker for dormancy release.

In almonds (Egea et al., 2003), phenological stages of flower buds were recorded *in vitro* in order to identify the end of endodormancy. The authors observed the change in the development of phenological stages after forcing and explained it with increasing cold accumulation. The order of dormancy release in the controlled condition was matching with the order of flower opening observed in the field. Moreover, pistil elongation was studied in almonds, apricots and peaches (Szalay et al., 2006). In apricots (Szalay and Németh, 2010), the dormancy status of the flower buds was identified by the changing characteristics of pistil growth rate. Also, this trend has also been seen in sweet cherries (Fadón et al., 2018).

In the present work, the flower bud development of 25 selected almond genotypes representing a wide range of flowering time was examined by three methods: observation of microsporogenesis, pistil growth during the dormancy period and forcing of shoots during three consecutive years. The aim of the study was to find differences among almond accessions regarding flower bud development. We also wanted to compare the three methods regarding the reliability in forecasting dormancy release.

MATERIALS AND METHODS

Plant material was obtained from the collection of almond genetic resources of the Hungarian University of Agriculture and Life Sciences (HUALS), Fruit Research Centre, Érd Elvira Major, Hungary, and studied between November and March, in 2019/20, 2020/21 and 2021/22. The yearly mean temperature is 9.9–10°C, and the mean temperature of the growing season is 16.7–16.9°C. The number of sunny hours per year is 1950 h, and the yearly precipitation is 550–570 mm. The soil is tsernozyom with 5% total lime content and 2.3%–2.5% humus (Ambrózy and Kozma, 1990). The experimental orchard was planted in 1996. The spacing is 7 × 3 m. The orchard has no irrigation, and the trees are on GF-677 rootstock. Our collection includes Hungarian landraces and cultivars (old and novel). Among 25 accessions used, five ('Budatétényi 70', 'Tétényi keményhéjú', 'Tétényi rekord', 'Tétényi bőtermő' and 'Tétényi kedvenc')

are commercial almond cultivars widely grown in Hungary, five ('Soleta', 'Belona', 'Vairo', 'Constanti' and 'Marinada') are Spanish commercial cultivars. The remaining 15 numbered accessions are landrace selections around the hills of Bakony collected in the 1960s.

Microsporogenesis studies

Three twigs with 1-year-old laterals were collected weekly from each accession every year. In the laboratory, 10 flower buds per accession were selected randomly. The anthers were removed by a tweezer, stained with carminic acetic acid and squash preparations were made for microscopic studies. The microspore development stage (archesporium, string, pollen mother cells [PMC], tetrad cells, microspores and pollen cells) of each microspore was recorded. The proportion of each stage was calculated by accessions and sampling dates. On the basis of weekly data, we estimated when 50% of the stages occurred and this calendar date was regarded as the transmission date from one stage to another. An accession having at least 50% of its flower buds in the string stage is regarded as reaching the end of endodormancy.

Pistil length measurements

Pistils of the 10 flower buds per accession that were used in microsporogenesis studies were examined. The length of the pistil was recorded on the microscope slide equipped with a stage micrometer (Carl Zeiss, Germany), with an accuracy of 0.1 mm. The resumption of pistil growth after being constant was considered as endodormancy release.

Method of forcing

Three twigs with 40–50 flower buds of each accession were collected weekly, from the beginning of November until the end of February to the beginning of March. One twig with 10–20 flower buds was taken as one replication. The twigs were transferred to the laboratory and immediately placed in water with their basal end cut in 1-L containers with 0.5 L of water and kept at room temperature with natural photoperiod reflected through the window. After 10 days, the number of open flowers was recorded. The date when accessions had 50% of open flowers was regarded as the end of endodormancy.

Data analysis

To evaluate the similarity of the accessions on microsporogenesis, hierarchical cluster analysis (with squared Euclidean distance and Ward's agglomeration method) as well as K-means clustering were performed using the length data of each developmental stage of microsporogenesis.

The endodormancy release dates determined by the three methods (microsporogenesis, pistil length measurements and forcing shoots) were compared using two-way Multivariate Analysis of Variance (MANOVA) with factors 'year' and 'accession' where the length of string and archesporium stages were used. Normality of the variables was checked with Shapiro–Wilk's test ($p > 0.25$).

Pair-wise comparisons were performed by Games-Howell's *post hoc* test in the case of pistil method and by Tukey's *post hoc* test in other cases in order to differentiate among accessions and find the year effect.

The statistical analyses were performed using the R statistical programme version 2.1 (R.CoreTeam, 2021).

RESULTS

Microsporogenesis studies

The results showed that flower buds of almond accessions underwent the classical developmental stages of microsporogenesis as described in Materials and Methods (Figures 1–3).

In 2019/20, the development of the archesporial tissue ranged between 27 and 64 days from the 1st of November (Figure 1). The string stage, which was marked as the beginning of microsporogenesis process lasted 18–23 days. This stage indicates the transition from the endodormancy to the ecodormancy phase. The transition periods of the PMC and tetrads were short; the anthers remained in the PMC stage from 6 days to 10 days and likewise in the tetrads from 8 days to 11 days depending on the almond accessions. The microspore stage began around 30–45 days after the start of the microsporogenesis process. The transition of the microspore stage lasted between 37 days and 44 days. The end of microsporogenesis (ecodormancy) was between March 9 and April 5. These indicated clearly that the process of microsporogenesis began around 90–100 days before flowering depending on accessions. The accessions differed in the developmental rate of microsporogenesis in particular in showing an important variation in the amount of time taken for the archesporium stage to differentiate into the string stage of microsporogenesis. At later stages of microsporogenesis, the transition periods became shorter and the variation increased during the whole process of microsporogenesis.

In 2020/21, the period of the development of archesporial tissue to produce string cells was shorter compared to the first year, in particular, for the earliest types as the development of the archesporium stage was noted after 15 days from the establishment of dormancy (Figure 2). However, for the case of the latest two cultivars 'Vairo' and 'Constanti', it remained almost the same. The string stage lasted 9–36 days, while the transition periods of PMC to tetrads and then tetrads to microspores lasted 6–13 days and 8–9 days, respectively. This means that the microspore stage began around 20–60 days after the start of the microsporogenesis process. The period of the development of microspore cells to produce pollen was relatively longer for most accessions compared to the previous season. This stage lasted between 29 days and 83 days. Consequently, the

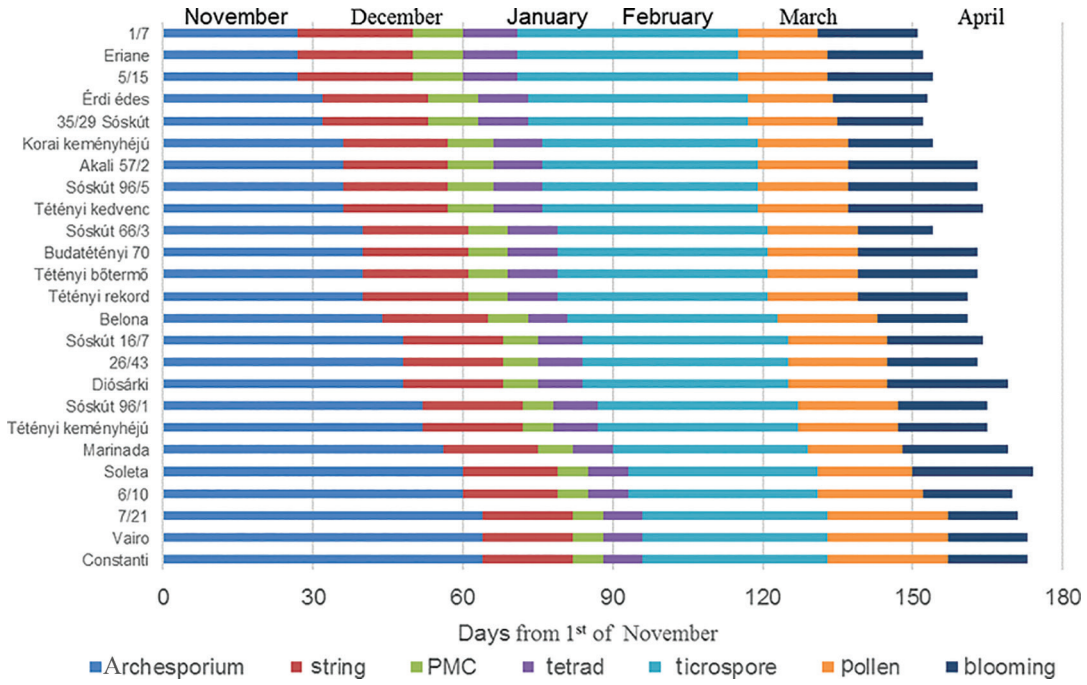

Figure 1. Phenological stages of microsporogenesis and blooming of almond cultivars, 2019/2020. PMC, pollen mother cells.

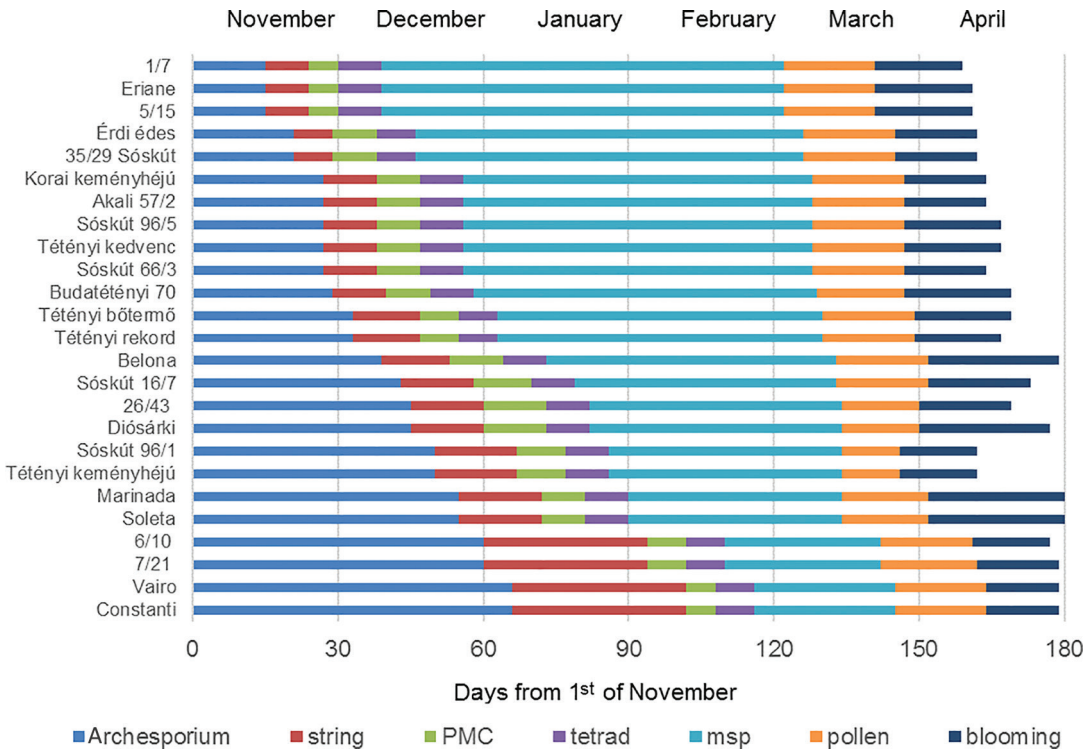

Figure 2. Phenological stages of microsporogenesis and blooming of almond accessions, 2020/2021. PMC, pollen mother cells.

end of microsporogenesis was extended by 5–22 days as the start to blooming date was between March 22 and April 14.

In 2021/22, the speed of microsporogenesis was comparable to that of 2020/21. The development of the archesporial tissue ranged between 14 days and 65 days during this year. However, the transition periods of the string stage were quite short for all the accessions as they lasted between 5 days and 8 days only (Figure 3). The transition periods of PMC and from tetrads to

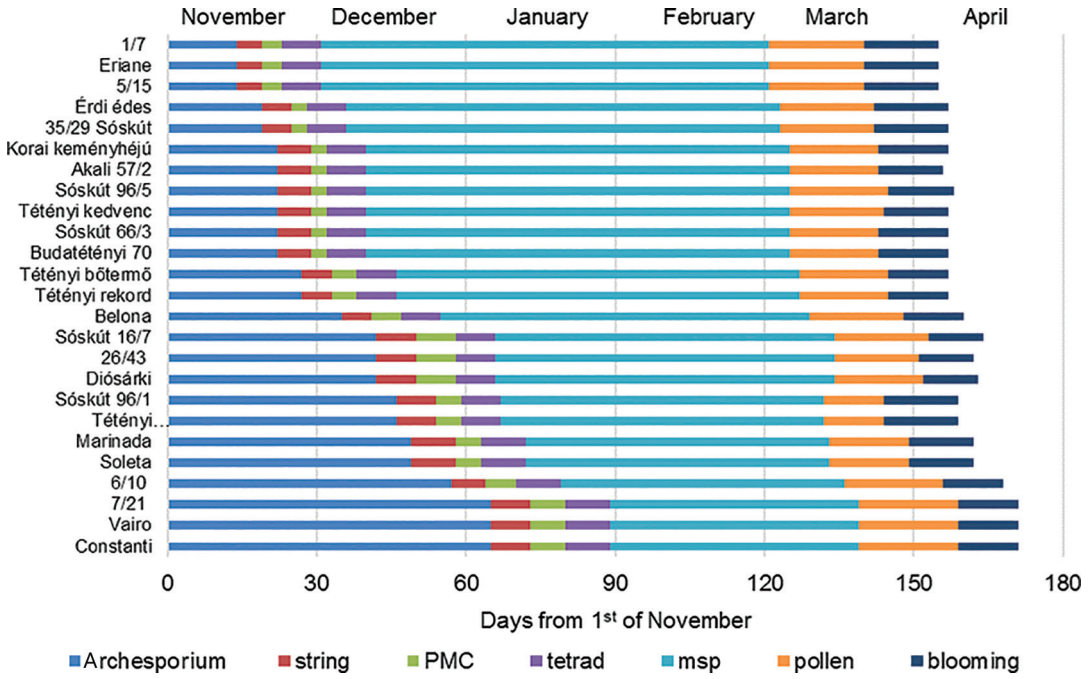

Figure 3. Phenological stages of microsporogenesis and blooming of almond accessions, 2021/2022. PMC, pollen mother cells.

microspores lasted 4–8 days and 8–9 days, respectively. This explains that the microspore stage began around 17–24 days after the start of the microsporogenesis process. But, the development of microspores cells to produce pollen was much slower in this year compared to both the other years as it lasted between 50 days and 90 days depending on accessions. The pollen cells were noticed 19–20 days before the end of microsporogenesis as blooming started between March 21 and April 9. In this year, the process of microsporogenesis began about 90–130 days before flowering similar to that in 2020/21.

As a summary, among all the studied accessions, '1/7', 'Eriane' and '5/15' had the shortest period of archesporial in the three studied years. The development of archesporial tissues of these accessions took 27, 15 and 14 days and the development of the microspore stage through tetrads took 71, 39 and 31 days in all three during 2020, 2021 and 2022, respectively. The accession '1/7' came to the end of microsporogenesis process on the 9th of April, 2 days earlier than 'Eriane' and '5/15' in 2020. However, in 2021 and 2022, all the three accessions had their end of microsporogenesis (ecodormancy) on the same day, i.e. on the 22nd or the 21st of March, respectively. Accessions '7/21', 'Constanti' and 'Vairo' had the longest archesporium stage (64, 66 and 65 days in all) during 2020, 2021 and 2022, respectively. The development of PMC to produce the microspores through tetrads took 96, 116 and 89 days in that order. In the flower buds, the final form of pollen grain was noticed 19–24 days before blooming; the blooming started on the 5th, 14th and 9th of April in 2020, 2021 and 2022, respectively.

According to the statistical results, the accessions showed significant differences in their total length and in each developmental stage of microsporogenesis ($p < 0.001$). Accordingly, accessions were classified based on developmental rates of microsporogenesis and the dendrogram generated by hierarchical cluster analysis with the Ward's method presented in Figure 4, where 5 main considerable groups and 10 subgroups can be observed for each year. In Figure 4, the green group contains the earliest accessions while the purple consists of the latest ones. The red group can be called 'early accessions' while the orange and navy groups are of the 'medium' and 'late accessions' groups, respectively. We can see that '1/7', 'Eriane' and '5/15' were grouped into the same 'earliest' group in all the 3 years while '35/29 Sóskút' and 'Érdi édes' were classified into the earliest group in 2020 and 2021, but into the 'early' group in 2022, which shows the potential climate sensitivity of these two accessions. Note that the K-means clustering with four groups revealed almost the same clustering output as the introduced hierarchical one with the only difference that '35/29 Sóskút' and 'Érdi édes' were classified into the 'earliest' group in 2022 too. 'Korai keményhéjú', 'Akali 57/2', 'Sóskút 96/5', Tétényi kedvenc', 'Sóskút 66/3', 'Budatétényi 70', 'Tétényi bőtermő' and 'Tétényi rekord' were grouped as early accessions in all the 3 years.

'Belona', 'Sóskút 16/7', '26/43'and 'Diósárki' belong to the medium accessions group while 'Sóskút 96/1', 'Tétényi keményhéjú' and 'Marinada' are the late accessions. 'Soleta' was classified as medium accession in 2021 and 2022, while 'latest' in 2020. In this difference, K-means and hierarchical clustering agreed

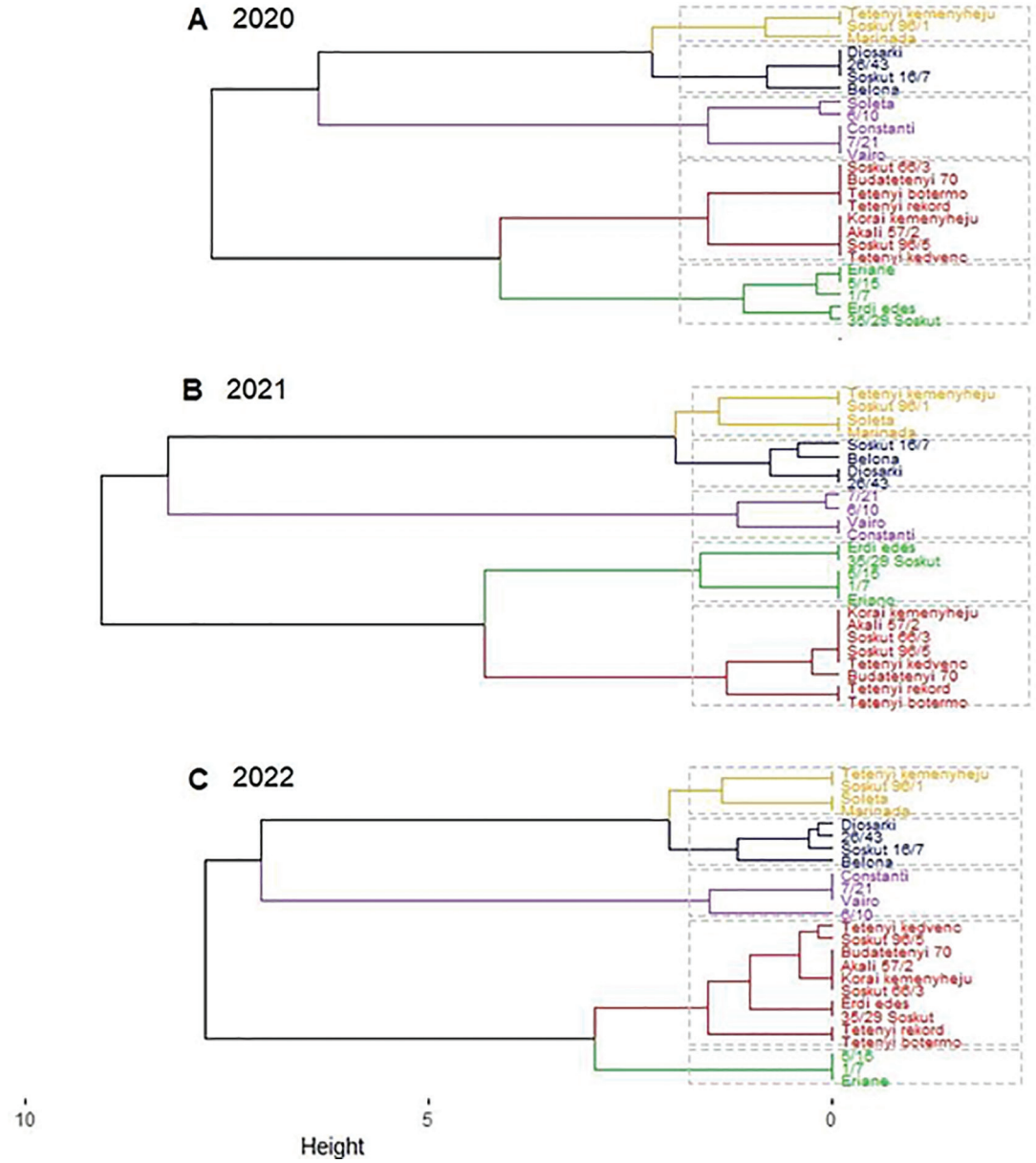

Figure 4. Almond accessions categorized on the basis of the rate of microsporogenesis in 2020, 2021 and 2022.

which refers to the climate sensitivity of accession 'Soleta'. Together with accessions '7/21', 'Constanti' and 'Vairo', '6/10' was also classified in the 'latest' group in all the 3 years by both classification methods.

Pistil length measurements

Before the plants entered the endodormancy phase, the growth was continuous and consistent (data not shown). Growth was arrested in the endodormancy phase; the increment was not apparent, with an average length of 1 mm (Figure 5). Pistil growth resumed when archesporium tissues in the anthers of the flower buds differentiated into the string stage, which was considered as a transition from endodormancy to the ecodormancy phase, first at a very slow increment followed by a few days of highly concentrated growth prior to blooming. This indicated that the accumulation of a certain amount of cold required by a cultivar is a prerequisite for pistil growth resumption. The accessions had considerable variations in their pistil growth rate particularly between the early and late types. The growth increment rate after resumption was more rapid of the latest ones than those of the earliest.

In 2019/20, the almonds presented pronounced variation in their pistil growth rate from the end of November onwards (Figure 5A). On the 27th of November for 'Eriane' and '5/15,' after a time period of steady 1 mm length, the rate of growth appeared to increase dramatically to 1.4 mm, after the necessary amount of chill had been accumulated. Also, the pistil length of '1/7' increased slightly at this time although the visible increment was recorded on the 7th of November. This change in pistil growth characteristics was seen after the 16th of January for the latest ones such as '7/21', 'Vairo' and 'Constanti'.

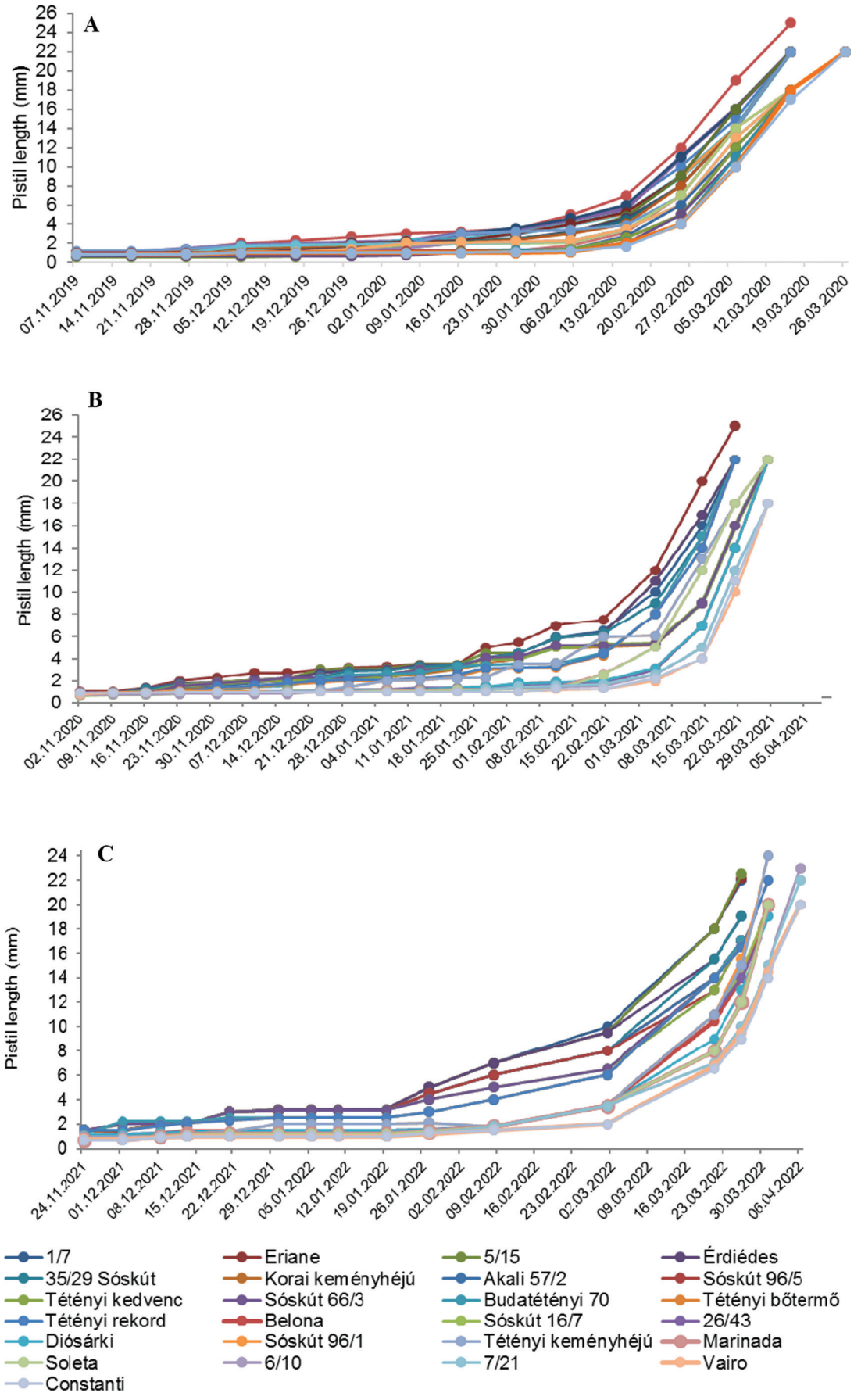

Figure 5. Changes in pistil length of 25 almond accessions on long shoots during winter in 2019/20 (A), 2020/21 (B), and 2021/22 (C).

In the years 2020/21 and 2021/22, the resumption of pistil growth of those earliest ones occurred on the 16th and 24th of November, respectively. In the case of the latest ones, pistil growth was arrested until the 19th of January in 2020/21 (Figure 5B), while in 2021/22, their growth was arrested further up to the beginning of February (Figure 5C). By that time, the earliest accessions were already with pistil lengths between 4.5 mm and 5.5 mm. In that pistil growth stage, the microspores were distinguishable in the anthers of those earliest ones. Of those earliest accessions, the resumption of pistil growth appeared at the moment when in the anthers of the flower buds, full string stage was distinguishable. While of those latest ones, the pistil appeared to resume growth at the moment when the separate PMC and tetrads in the anthers of the flower buds were distinguishable.

Flower bud development under the forcing conditions

The developmental rates of bud shoots exposed to forcing conditions were clearly affected by accessions and yearly climatic conditions. The flower bud development overlapped into 10 groups with slight differences of overlapping each year (Figure 6). In the year 2019/20, flower buds of the earliest accessions 'Eriane', '1/7' and '5/15' started to present open flowers in the first week of November (Figure 6A). The developmental rates were gradual. On the 17th of November, about 22% of the buds presented open flowers. Twenty days later, on the 7th of December, the amount reached 50% and 100% on the 16th of January. By that time, they reached 50% of open flowers and in the anthers of the flowers, 90% of the string was distinguishable. Flower buds opened 100% following the appearance of the microspore stage and pistil growth was already resumed.

In the case of the latest accessions '7/21', 'Vairo' and 'Constanti', flower bud opening started after the 6th of January. In the anthers of the flower buds, separate PMC of microsporogenesis stages were distinguishable at this moment. Once started, flower bud opening increased at higher rates as indicated by steep slopes of the sigmoid lines. Of those latest ones, the proportion of opened flowers reached half in the first week of February and 100% towards the end of the month. This was 30 days before flowering under field conditions. During this year, the flower buds of forced shoots for most of the studied accessions presented around 50% open flowers with the appearance of tetrads and microspore stage.

In the year 2020/21, flower buds of the earliest accessions began to present open flowers after the 1st of December, and 15 days later, about 50% of the buds presented open flowers (Figure 6B). The flower bud opening began with the appearance of tetrads and in the anthers of the flower buds. The end of the reduction process was already indicated by the occurrence of the microspore stage at the time when about 50% of the buds presented open flowers. For the case of the latest ones, '7/21', 'Vairo' and 'Constanti', flower bud opening began after the 6th of January with the string stage formation. Around 50 days later, on the 26th of February, the proportion of opened flowers reached more than half when in the anthers of the flower buds the microspores and the first change of pistil length increment rates were noticeable. Flower buds of forced shoots began to present open flowers with the appearance of tetrads and presented around 50% open flowers with the appearance of a microspore stage similar to that of 2019/20. However, with some accessions, flower bud opening started with the transition to the string stage and presented more than half-open flowers with the development of tetrads.

In the year 2021/22, the development of flower buds under the forcing started a bit earlier than in 2020/21 but similar to that of 2019/20. The rate of flower bud opening was quicker in this year than in the years 2019/2020 and 2020/21 (Figure 6C). On the 15th of November, about 25% of the buds of the earliest accessions 'Eriane', '1/7' and '5/15' presented open flowers when the archesporium tissue in the anthers of flowers differentiated to the string stage. Seven days later, the flower buds presented 50% open flowers with the formation of tetrads. By that date, the flower buds of these accessions presented around 38% and 1% of open flowers in 2020 and 2021, respectively. This is probably due to differences in environmental factors observed in the 3 years that influenced the response of buds to forcing temperatures and flowering date in spring. For most of the studied accessions, the flower bud opening started with the formation of the string stage and reached around 50% open with the appearance of separate PMC and tetrads during this year. This is probably due to differences in environmental factors, especially temperature.

After all, the dates of endodormancy determined by microsporogenesis, pistil length measurements and forcing shoots were compared using two-way MANOVA with the factors 'year' and 'accession'.

The overall MANOVA resulted in significant accession and year effect (Wilk's lambda = 0.006 and 0.023, respectively, both with $p < 0.001$).

The follow-up univariate Analysis of Variance (ANOVA) revealed highly significant genotype and year effect for all the three methods (accession: F [24; 48] > 28.8; year: F [2; 48] > 13.71, all with $p < 0.001$). *Post hoc* test results show that all three methods were able to differentiate the accessions, however, showed different resolutions and slightly different classifications of the accessions (Table 1). Most groups (eight) was found among microsporogenesis studies, whereas three groups were formed by pistil length and two forcing method. They all agree that accessions '1/7', 'Eriane' and '5/15' are significantly earlier than all the others. However, based on the pistil method, accessions '35/29 Sóskút', 'Érdi édes', 'Akali 57/2'and 'Tétényi bőtermő' also appear among the earliest. Similarly, according to pistil length measurements, the accessions '6/10', 'Soleta', '7/21', 'Constanti' and 'Vairo' are significantly

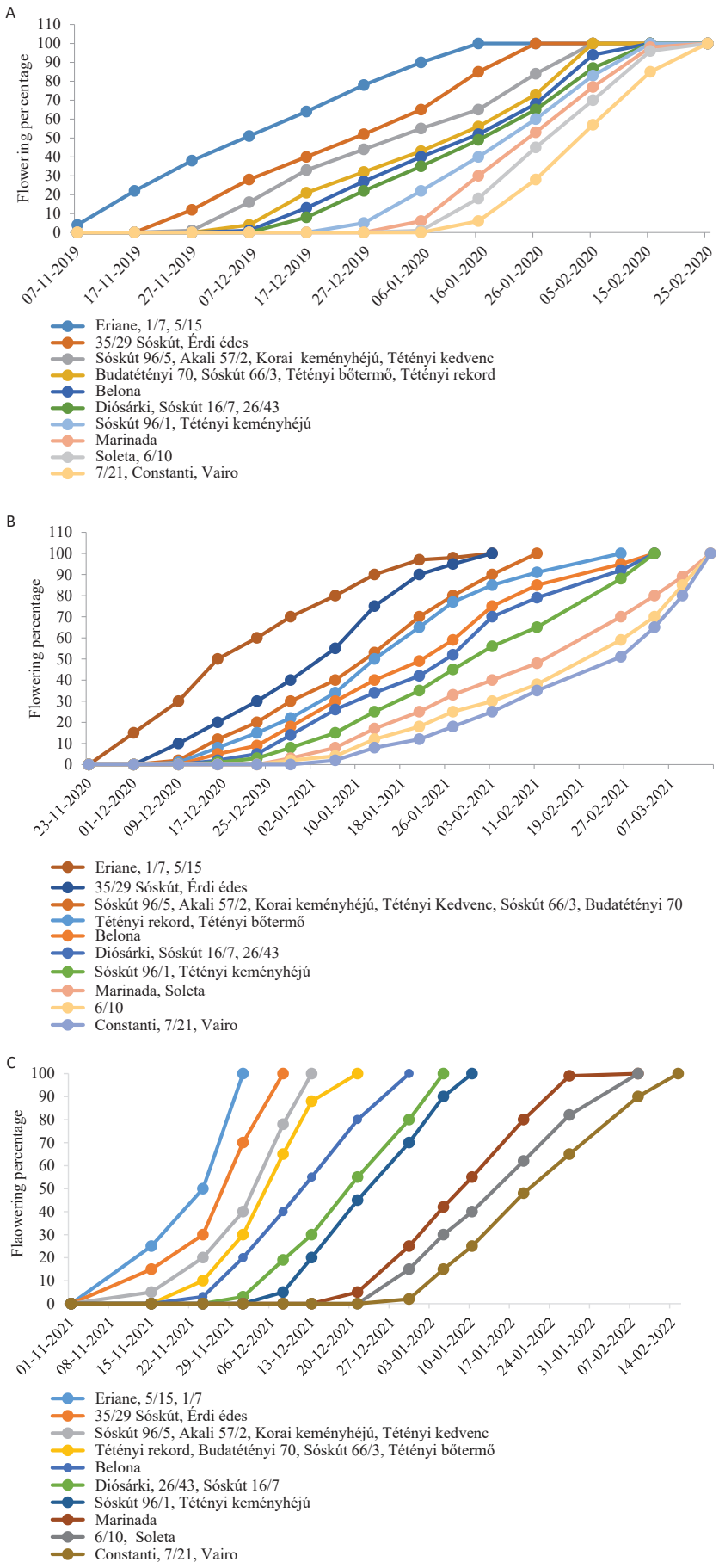

Figure 6. Flower bud development observation in controlled forcing conditions in 2019/20 (A), 2020/21 (B), and 2021/22 (C).

Table 1. Average length of endodormancy (days) based on microsporogenesis, pistil and forcing methods for the 3 years.

Accessions	Methods		
	Microsporogenesis	Pistil	Forcing
5/15	18 ± 6.66 a	21 ± 5.69 a	35 ± 11.06 a
1/7	18 ± 6.66 a	25 ± 10.59 a	35 ± 11.06 a
Eriane	18 ± 6.66 a	21 ± 5.59 a	35 ± 11.06 a
35/29 Sóskút	24 ± 6.43 ab	28 ± 16.09 a	48 ± 18.36 ab
Érdi édes	24 ± 6.43 ab	21 ± 5.69 a	48 ± 18.36 ab
Akali 57/2	28 ± 6.03 abc	25 ± 4.93 a	54 ± 20.42 ab
Korai keményhéjú	28 ± 6.03 abc	30 ± 13.58 ab	54 ± 20.42 ab
Sóskút 96/5	28 ± 6.03 abc	25 ± 4.93 a	54 ± 20.42 ab
Tétényi kedvenc	28 ± 6.03 abc	33 ± 20.31 ab	54 ± 20.42 ab
Sóskút 66/3	29 ± 8.74 abcd	29 ± 14.57 ab	59 ± 20.50 ab
Budatétényi 70	30 ± 88.54 abcd	30 ± 4.04 ab	59 ± 20.50 ab
Tétényi bőtermő	33 ± 6.03 abcde	28 ± 4.36 a	59 ± 21.08 ab
Tétényi rekord	33 ± 6.00 abcde	37 ± 18.34 ab	59 ± 21.08 ab
Belona	39 ± 4.51 bcdef	53 ± 14.64 ab	67 ± 22.75 ab
Sóskút 16/7	45 ± 2.89 cdefg	53 ± 14.73 ab	71 ± 19.70 ab
26/43	45 ± 2.52 defg	52 ± 12.70 ab	71 ± 21.17 ab
Diósárki	45 ± 2.52 defg	37 ± 5.00 ab	71 ± 19.70 ab
Sóskút 96/1	46 ± 2.65 efgh	54 ± 3.46 abc	72 ± 16.74 ab
Tétényi keményhéjú	46 ± 2.65 efgh	64 ± 19.29 bcd	72 ± 17.35 ab
Marinada	53 ± 3.46 fgh	89 ± 9.29 cd	85 ± 16.50 ab
Soleta	54 ± 5.03 fgh	93 ± 6.56 d	88 ± 13.50 ab
6/10	58 ± 3.03 gh	93 ± 6.56 d	91 ± 18.68 ab
7/21	63 ± 2.52 h	91 ± 7.23 d	97 ± 19.01 b
Constanti	65 ± 1.16 h	96 ± 8.51 d	97 ± 19.22 b
Vairo	65 ± 1.16 h	97 ± 5.69 d	97 ± 19.22 b

Variables represent the mean of replications ± their corresponding standard deviation. Means having same letter(s) in a column are not significantly different according to two-way ANOVA followed by Tukey's *post hoc* test ($p \leq 0.05$).

later. But based on the other two methods, accessions '7/21', 'Constanti' and 'Vairo' are the significantly later ones.

As for the year comparisons, *post hoc* tests agreed that the year 2021/22 resulted in the earliest development based on all the three methods. However, while in the case of microsporogenesis method, the development was significantly earlier in this year than in 2020/21, and in the year 2019/2020, it was significantly later than both of the other years; the order was different in the case of forcing method: the significantly latest year was 2020/21. In the case of pistil method, years 2021/22 and 2020/21 did not differ significantly, while 2019/2020 resulted in significantly later development.

DISCUSSION

According to our results, anthers of flower buds remained at the same stage of development during endodormancy, characterised by the appearance of archesporium tissues, with a pistil having no visible growth. At winter dormancy release, archesporium tissues of anther differentiated into PMC and the pistil growth rate accelerated (Szalay, 2006). This stage of anther development has been also reported in other *Prunus* species (Szalay, 2006; Fadón et al., 2018, 2019). In apricot, many studies agree (Julian et al., 2009; Szalay and Németh, 2010; Andreini et al., 2012; Hajnal et al., 2013; Wu et al., 2019; Herrera et al., 2022) that the differentiation of archesporium tissues of flower buds has been related to the chilling fulfilment and with endodormancy release, an advanced microsporogenesis process was observed with the appearance of tetrads and pollen grain. The result of this work is in accordance with the reports of the authors.

The speed of flower bud development differed depending upon almond accessions but the dates and transition periods to each stage of microsporogenesis for a specific accession altered to some degree depending on yearly local climatic conditions, particularly the

temperatures. The climate sensitivity of almonds is not the same. In previous studies (Lamp et al., 2001), variations in the time of flower bud development of almonds occurred among locations, years, within and among almond accessions. The same result has been revealed in apricots (Bartolini et al., 2006b; Hajnal et al., 2013; Szalay et al., 2019). Our results show that low temperatures during the endodormancy period had an important microsporogenesis process advancing effect, whereas extended periods of low temperatures during the microsporogenesis process of the ecodormancy phase had a delaying effect on the development of pollen grains and consequently, on bloom dates of almonds. Similar results have been reported by Citadin et al. (2001); Harrington et al. (2010); Scalabrelli and Couvillon (1985). The reason is that, cold temperatures during ecodormancy delay flower bud development as longer time is required for the buds to accumulate their heat requirements. But heat accumulation may take place much faster towards early springs and can speed up the change in flower bud development (Lamp et al., 2001; Alonso et al., 2005; Szalay and Németh, 2010). On the other hand, the mild winter during the ecodormancy of the microsporogenesis process accelerated the flower bud developmental rate and decreased frost hardiness.

Flower bud developmental rate emphasising special attention on determining endodormancy release has been the focus of great interest in this field of research for the quantification of chilling units and the growing degree hours needed of a given cultivar (Szalay et al., 2019). Chilling unit and the growing degree hours requirements of the cultivar determine its adaptation to specific ecological conditions (Bassi et al., 2006; Julian et al., 2009; Herrera et al., 2022). However, the lack of a standard method to establish the end of endodormancy makes it difficult to know if a given cultivar is adapted to a specific region based on its chilling unit and heat requirements (Herrera et al., 2022). According to different authors (Bartolini et al., 2006b; Andreini et al., 2012), dormancy release of *Prunus* species was marked with the appearance of tetrads or male meiotic division. The result observed from this study clearly agreed with the cited view of (Szalay and Németh, 2010; Fadón et al., 2019; Szalay et al., 2019) that the development of the string stage was marked as a potential biomarker for flower bud dormancy release using the microsporogenesis method. Regarding the pistil length study, a length increment of steady 1 mm length was considered as a mark for endodormancy release. Flower bud development under the forcing conditions is also related to chill accumulation as the forcing of shoots before the start of winter produced no flower. Maneethon et al. (2007) reported that flower bud development under forcing depends on accumulated chilling units in winter. For most of the almond genotypes, flower bud opening appeared to reach 50% and above when the meiotic cell division had already been completed in the anthers of the flower buds. This occurred much later than the endodormancy was believed to be released based on both microsporogenesis and pistil methods. In peaches, the closely related species to almonds, it has been reported that the artificial warm temperatures were ineffective in showing the endodormancy breaking date for cultivars with high chilling requirements (Bartolini et al., 2006b). A good correspondence was found between the appearance of pollen tetrads of low chilling required cultivars and endodormancy breaking date using the bud weight method.

Selection of genotypes with slow phenological development and a high chilling unit requirement of more than 1000 h is a possible way of avoiding spring frost damages in cold regions (Szalay et al., 2006). In our case, the best genotypes could be '7/21', 'Constanti'\ and 'Vairo'. These genotypes had slow development, high chilling and heat requirements and thus, they are not in danger of spring frosts. But, it is the reverse in warm areas where there is the problem of insufficient chilling to overcome dormancy. The frost hardiness of genotypes was also examined in artificial freezing temperatures and genotypes with faster floral bud developmental rates were exposed to the risk of frost.

CONCLUSIONS

This is the first detailed report on flower bud development of almonds that has been described by three methods.

From the results of our research, it can be concluded that flower buds significantly affected by the accessions as well as the years studied. It proves that choosing the appropriate cultivars for a given growing site is crucial. Flower bud development can be examined accurately by using the microsporogenesis method to better understand the transitional changes occurring throughout the different phases of flower bud development, from bud formation in the summer to flowering in the following spring. The string stage could be indicated as a suitable marker for the determination of dormancy breaking date. Measurement of pistil length was good enough to give a clear indication of the dormancy status of the almonds but less accurate than the microsporogenesis method. With regard to the estimated time the almond overcame dormancy, as the day, 50% of open flowers using buds of sample shoots exposed to forcing conditions did not seem accurate. But, the differences among the genotypes still remain noticeable. Almonds with a short endodormancy period were found to be quick in their floral bud developmental rate.

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AUTHORS' CONTRIBUTION

KBT designed the study of the work, conducted the laboratory and field, and wrote an original draft of the manuscript. LS and ZB had inputs in the design of the work and reviewed the manuscript. ML analysed the results and reviewed the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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