

Selection and development of late-flowering apple varieties to avoid frost damage in organic fruit growing

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Abstract

One way of avoiding damage regularly caused by late frost events in spring in pome fruit crops is to grow late-flowering varieties. If flowering takes place late enough, considerable yield losses can be avoided. There are studies that show that late flowering has already been the subject to research and breeding. However, there is still no variety on the market that flowers at such a late stage. This project is concerned with the selection, evaluation, and development of late-flowering apple varieties. A major focus is on testing a selection mode that allows indirect selection for late flowering before entering the adult phase.

Keywords: Pome fruit, late flowering, selection, breeding, varieties

Introduction

Damage to apple blossoms due to late spring frosts is causing massive yield losses in some years. Data collected at LVWO Weinsberg (Germany) over 60 years show that the flowering time of apple varieties nowadays is about 19 days earlier due to climate change (Rueß 2020). Several fruit research stations across Europe report similar observations (Legave et al. 2015). The risk of yield losses due to late spring frosts is therefore increasing (Rueß 2020). Growers have various technical tools at their disposal to reduce late frost damage to their crops. Frost protection irrigation, wind machines, frost candles and heating ovens are used, but they often involve high investment costs, a lot of maintenance cost and, depending on the type of frost, limited efficiency (Snyder & Melo-Abreu 2005). Another approach to solve this problem is to cultivate apple varieties with such a late flowering period that late spring frosts cannot cause damage to the blossom. Late flowering apple varieties were already identified by Schmidt (1940) and are part of various investigations and breeding approaches during the 20th century but none of these varieties meets the requirements of the dessert apple market. Early research at the Kaiser Wilhelm Institute in Müncheberg suggests that the time of leaf budbreak and flowering is genetically fixed and therefore heritable (Schmidt 1940). According to Murawsky (1967) the gene structure seems to be polygene and dominantly inherited. Later investigations at Müncheberg as well as at East Malling indicate, that there is a strong link between the time of leaf budbreak of the juvenile seedlings and the time of flowering of adult trees, suggesting, that the selection of late flowering seedlings can be successfully carried out during the second year of cultivation (Murawsky 1959, Murawsky 1967, Tydeman 1958). A study of Mehlenbacher & Voordeckers (1991) indicates, that the time of seed germination after stratification can be used as selection criterion as well, as they found a strong correlation between the time of seed germination and the time of leaf budbreak. The following objectives are being pursued as part of this project: 1) Search for frost tolerant, late flowering apple varieties and establishment of a gene pool. 2) Evaluation and selection of suitable varieties for organic

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farming and heirloom orchards. 3) Crossbreeding of late flowering new varieties by combining the late flowering trait with quality and resistance traits from modern varieties.

Methods

Search for frost tolerant, late-flowering apple varieties

Varieties that flower at least two weeks later than common apple varieties from the dessert fruit range ('Elstar', 'Topaz', 'Gala') are regarded as late flowering in this study (Figure 1.). The late-flowering 'Taffetapfel', which flowers about 4 weeks later than the standard varieties at the LVWO Weinsberg trialsite, served as a reference.

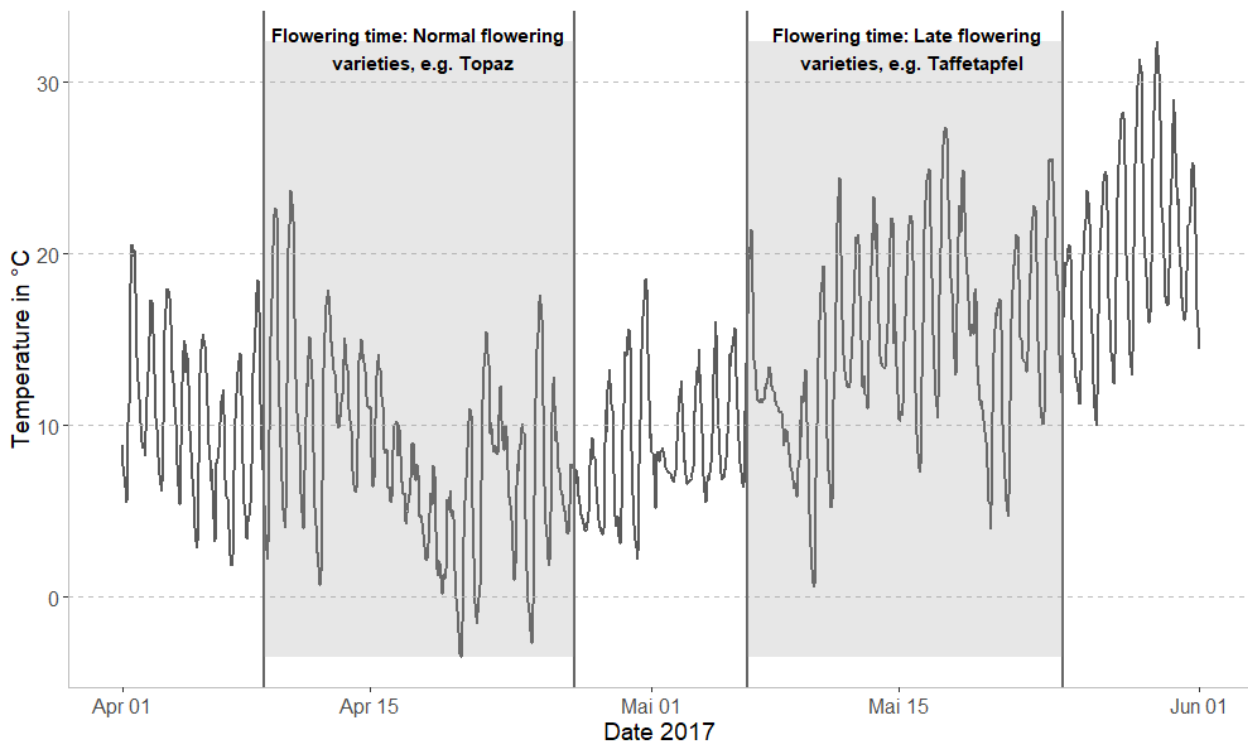


Figure 1: Temperature curve (hourly average measured at a height of 2 metres) and flowering periods of the normal-flowering variety 'Topaz' and the late-flowering variety 'Taffetapfel' at the LVWO trial site in the year 2017.

Crossbreeding and finding a selection mode

From 2020 to 2023, 23 crossbreeding combinations were carried out between late-flowering varieties and modern varieties and breeding lines with dessert apple quality as parent varieties (Table 1). The non-late flowering parents were chosen for their fruit quality and disease resistance (apple scab, powdery mildew) or robustness (fireblight).

Crossings are performed at different field orchard sites during the flowering period. After harvesting the fruits at maturity, all seeds were removed at the end of October, placed in jars with moist substrate (HAWITA Fruhstorfer® Aussaat- und Stecklingserde) and stored in a cold storage room at 4°C (stratification). After three months of stratification the jars were taken out of the refrigerator every week, the seeds that had already germinated were removed and planted in substrate in multi-pot plates and later transplanted into larger pots. The seedlings were raised in the greenhouse at ~24°C, supplied with a universal fertilizer (WUXAL® Super). At the beginning of May, the plants were moved to an irrigated outdoor container bed and planted to the field in the following autumn/winter. In order to find a selection mode, it was checked whether there is a correlation between the time of

germination, the time of leaf budbreak and flowering, as described in the above-mentioned literature. In addition, it was checked whether this correlation, if it exists, can serve as a selection criterion. To date, the germination dates of three crossing generations were recorded together with the leaf budbreak dates of two of these generations. For the oldest generations, the data from two seasons were evaluated. The recording of the leaf budbreak was done weekly, using a rating scale from 1 to 6, modified after Mehlenbacher (1991): 1 = dormant buds, 2 = green terminal bud, 3 = terminal- and lateral buds are green, 4 = light leaf budbreak, 5 = leaf budbreak < 3cm, 6 = leaf budbreak > 3 cm.

For statistical calculations, two different parameters were tested for their suitability. The mean value of the phenological stages (1-6) of a seedling recorded over the whole rating period and the number of days of the year when the seedlings reached stage 3 ("days to leaf budbreak") are highly correlated ($r=-0.9$, p -value < 0.01) and were therefore evaluated as useful values to represent the relative leaf budbreak time. To find out whether the germination date is related to the leaf budbreak date, and how the leaf budbreak date of the second year relates to the leaf budbreak date of the first year, correlations between these parameters using Spearman's rank correlation and additionally linear regressions were calculated. Further, a Kruskal-Wallis test was performed to evaluate whether the populations differ significantly for the distribution of the germination date and the leaf budbreak date. A pairwise Wilcoxon test, applying the adjustment method BH, was performed to represent the pairwise comparisons between the populations.

Genetic analysis

After analysing the leaf budbreak data (in the first season only) of the three mentioned populations, we selected in total 400 genotypes (DA = 91, DB = 110, DC = 176, other = 23 varieties) for the genetic analysis. The genotype pool included all seedlings that showed leaf budbreak later than the average timing of leaf budbreak calculated across the seedlings from all three populations. Other seedlings (early and moderate leaf budbreak) were selected randomly for genetic analysis until the total number of seedlings reached 400, which was the maximum of the sequencing capacity. Leaf samples were taken in June when the leaves were fully developed. Leaf disks from three distinct apple populations, alongside parent varieties, were punched out and immediately refrigerated. These samples were then sent to LGC Group (Teddington, England) for DNA extraction and subsequent SeqSNP analysis. SeqSNP represents a targeted genotyping method focused on previously known genetic variants. SeqSNP was based on prior genotyping research on apple budbreak, including both published literature and unpublished data provided by A. Peil and J. Lempe. Those genetic variations were annotated with 95% similarity to the double-haploid apple reference genome (GDDH13 Version 1.1), and these annotations were supplied to LGC for the SeqSNP sequencing, which entailed 150 bp paired-end Illumina sequencing on a NovaSeq 6000 platform. Post-sequencing, the raw data underwent demultiplexing and adapter trimming. Further, the data were aligned to the apple reference genome by the Burrows-Wheeler Aligner (<https://github.com/lh3/bwa>) and variants were called via "gStacks" (<https://catchenlab.life.illinois.edu/stacks/>). In R, for further data filtering we employed base R functions and packages such as 'VariantAnnotation', 'ape', 'SNPRelate', and 'ggplot2'. Initially the variant analyses that was performed after filtering out the sequences with missing nucleotides and separating populations did not yield expected results in phylogenetic trees or PCA, since the population structure could not be recovered. Consequently, a revised approach was adopted, converting variants into binary data representing the presence or absence of variations, which are the results presented herein. Variants with a frequency below 0.1 or equal to 1 were considered either rare or non-variant and were filtered out accordingly.

Results

Search for frost tolerant, late flowering apple varieties

The search for late flowering apple varieties resulted in a selection of 50 varieties, which are in a more or less late flowering period. Most of the varieties found are not of good dessert apple quality and are not suitable for commercial production. The varieties 'Christiansapfel' and 'Königlicher Kurzstiel' stand out due to their late flowering time and good fruit quality. The variety 'Königlicher Kurzstiel' was tested to have a good shelf life during an earlier project at the LVWO (Haug et al. 2021) and was rated very well for its taste during a juice tasting. A selection of 17 of the varieties are grafted and will be planted at the LVWO trial site. Well-founded, comparative variety descriptions and recommendations can only be given after the selected varieties have been evaluated under uniform conditions over several years.

Crossbreeding and finding a selection mode

The crossbreeding conducted during the years 2020 to 2022 yielded three generations of ~1000 seedlings all together until now. The results relating to the oldest seedling populations are presented here, as this is where most of the data was collected until now (Figure 2.). This population consists of the crossing population: DA = 'Heslacher Gereutapfel' x 'Flavia' (92 Seedlings), DB = 'Spaetbluehender Taffetapfel' x 'Flavia' (110 Seedlings), DC = 'Christiansapfel' x 'Flavia' (311 Seedlings).

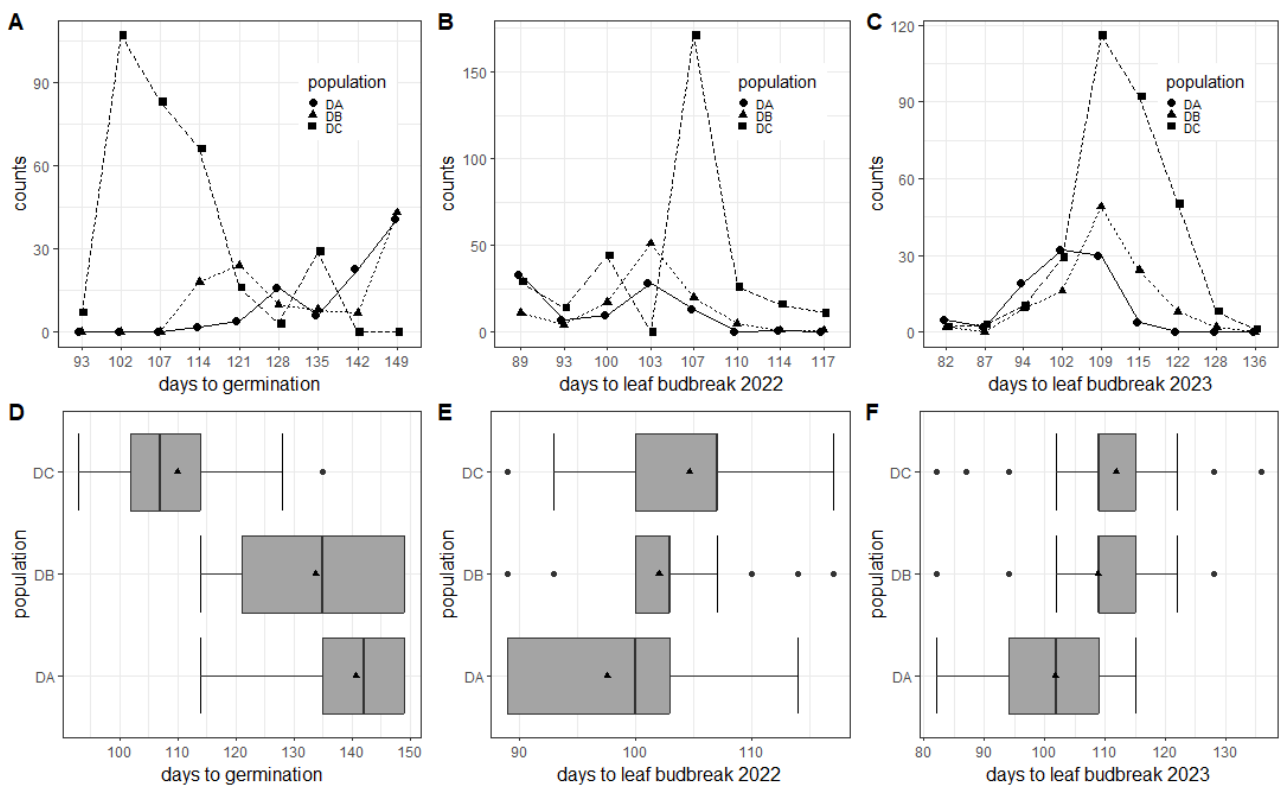


Figure 2: **A:** Plot of germination rates as "days to germination" counted from the date of the start of stratification, separately for each crossing population. **B** and **C:** Graphs showing leaf budbreak rates as "days to leaf budbreak 2022/23" counted from the first of January of each year for each population. **D:** Box-whisker plot showing the distribution of "days to germination" separately for the population. **E** and **F:** Box whisker plots showing the distribution of "days to leaf budbreak 2022/23" separately for each population. All box whisker plots contain the mean value, which is represented as a triangle.

Most of the seeds of the three population germinated within ~150 days from the beginning of the stratification, after this period the experiment was stopped.

The values of “days to germination” do not follow a normal distribution. This distribution was tested positively for significant differences between all three population using a Kruskal-Wallis rank sum test and a pairwise Wilcoxon test ($p < 0.005$). In particular, the DC population with ‘Christiansapfel’ as mother variety shows a very different germination behaviour compared to the other two population. Most of the seeds in this population germinated within the first four weeks of the germination study, with the highest germination rate achieved in the second week. The germination studies of the following two years show a similar tendency for crosses with ‘Christiansapfel’. This tendency appears to be stronger when ‘Christiansapfel’ is used as the mother variety. The distribution of the “days to leaf budbreak” approaches a normal distribution in both years. Here too, the distribution of the three population differs significantly from one another.

The correlation analysis between the variables “days to germination” and “days to leaf budbreak 2022” (Figure 4, left) results in a slightly negative correlation for all populations combined ($\rho = -0.32$, $p < 0.001$). Taking a closer look at the individual populations, these two variables are only positively correlated in the DA population ($\rho = 0.43$, $p < 0.001$). The analysis, testing whether “days to leaf budbreak 2022” is correlated to the “days to leaf budbreak 2023” results in a positive correlation of $\rho = 0.626$ ($p = 0.00$) for all populations combined (Figure 3, right). On closer examination of the individual populations, the positive correlation appears to be strongest for the DC population ($\rho = 0.62$, $p = 0.00$). The linear regression model confirmed that there is a significant positive correlation between “days to germination” and “days to leaf budbreak 2022” for the DA population, but only 10% of the variance of the variable "days to leaf budbreak" can be explained by the fitted model ($R^2=0.1$).

For the predictability of the leaf budbreak date by the leaf budbreak date of the previous year, the linear regression provides similar results to the correlation analysis. There is a clear positive regression coefficient between the two variables (DA: 0.49, DB: 0.58, DC: 0.69) for all populations. But the R^2 value is low for all populations, highest for DC ($R^2=0.36$).

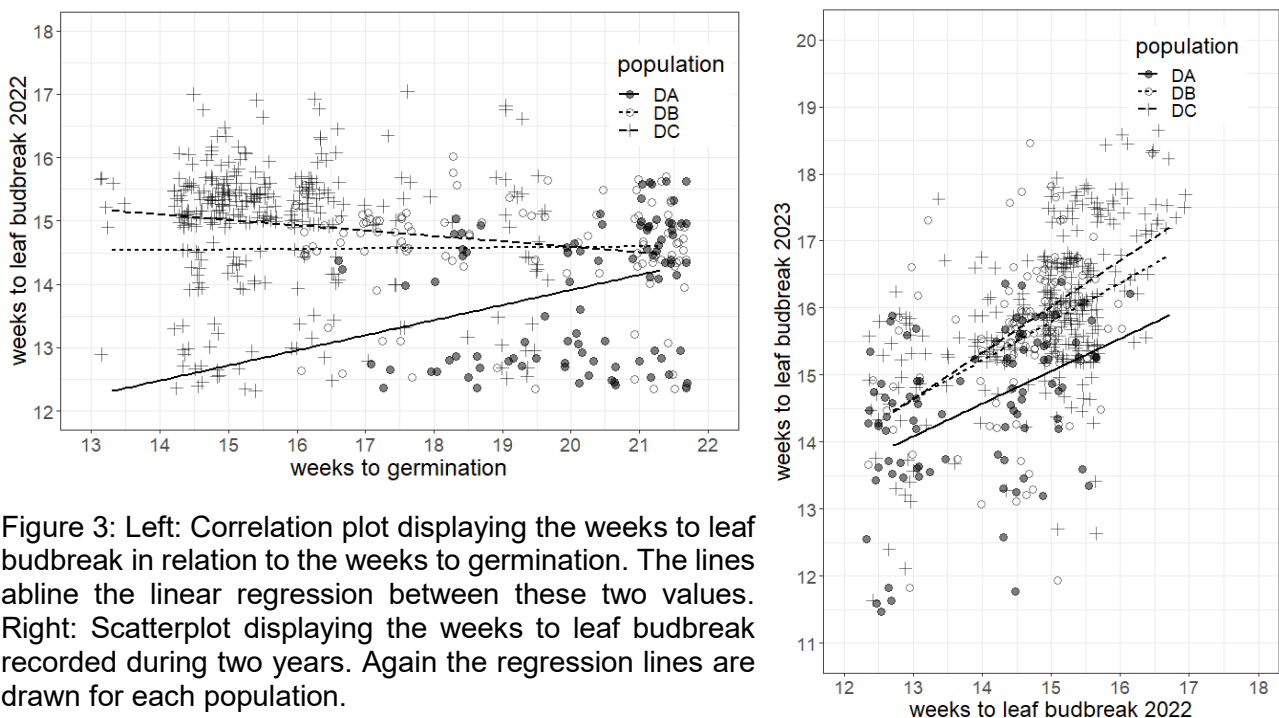
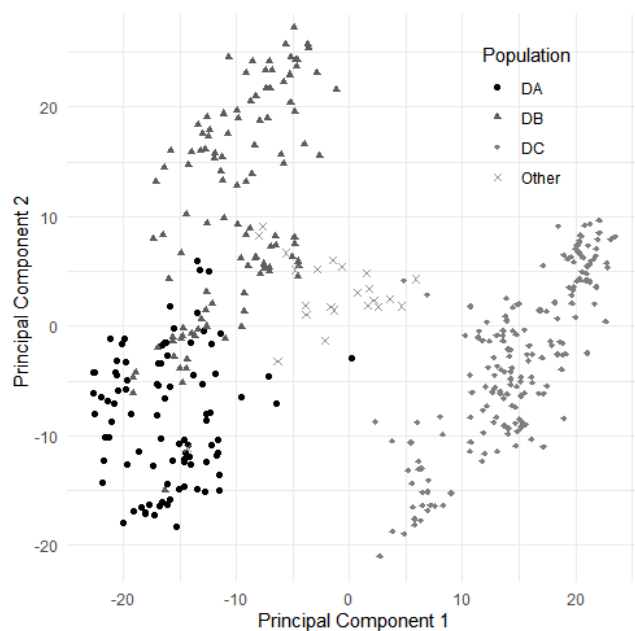


Figure 3: Left: Correlation plot displaying the weeks to leaf budbreak in relation to the weeks to germination. The lines abline the linear regression between these two values. Right: Scatterplot displaying the weeks to leaf budbreak recorded during two years. Again the regression lines are drawn for each population.

Genetic analysis

The SeqSNP sequencing procedure resulted in a total number of 86,605,644 reads, achieving an average effective coverage of 249x per target. Post-filtering, the targets lead to a total of 1,554 variants, averaging 3 variations per target. This statistic underscores the efficacy of the target selection. The PCA analysis (Figure 4) illustrates the distribution of the 23 varieties (“Other”) and three breeding lines DA, DB, and DC. The first principal component (PC1) distinctly separates population DC from the others and aligns DA and DB on the left, PC2 primarily differentiates between DA and DB. This separation suggests a higher degree of genetic relatedness within these populations compared to these populations and DC. The widespread distribution of all populations indicates a diverse heterozygotic genetic background among the parent genotypes. This diversity forms a robust foundation for the forthcoming phase of the project - an association study that aims to draw conclusive correlations between genetic variation and phenotypic traits.

Figure 4: Principal component analysis (PCA) of the populations (DA, DB, DC) and varieties ('Other'). Principal component (PC1), accounted for 13% of the genetic variation and PC2 accounted for 7% of the variation. The PCA has been performed on data from a targeted sequencing approach – SeqSNP – based on previously identified genetic variations that could be correlated with vegetative or generative bud break.



Discussion and Conclusion

The results of the germination test show, that the combination of the parents has a significant influence on the offspring “days to germination” and “days to leaf budbreak” distribution. The leaf budbreak of the seedlings in the field recorded during the spring 2022 and 2023 happens over a time span of 4-8 weeks and follows nearly a normal distribution. This can be an indication that the underlying inheritance mechanism is that of a quantitative trait (Miedaner 2010). According to the presented results, it is not possible to confirm the assumption that the germination date could be used as indirect selection criterion for genotypes with a late leaf budbreak date, as there is no clear positive correlation of these two variables. In case of the DC population these two variables are even negative correlated. The R^2 value of the linear regression can be regarded as part of the variance which is fixed by genetic effects and for this repeatable. As R^2 is highest for the DC population, it can be assumed that the cross with the ‘Christiansapfel’ as mother variety produces the offspring with the most environmentally stable relative budbreak date. Further, as ‘Christiansapfel’ produces the highest proportion of seedlings with a late budbreak date, it seems, that this variety is a good crossing parent to inherit the late budbreak trait to its offspring. However, as this study only includes data from two years, this result must be treated with caution and verified in further studies.

Assuming that the cross progeny is in the range between the early and the late parent with regard to the leaf budbreak date, a selection mode as follows would be conceivable: The seedlings are rated in the second year of vegetation according to their leaf budbreak date. The sharpness of selection can be determined on the basis of a fixed budbreak delay measured from the start of budding of the earliest seedlings. Applied to the analysed seedling population using a delay of three weeks, only ~9% of the DC population, 1% of the DA population and ~2% of the DB population would remain. In this scenario nearly all seedlings from the DA and DB population would be lost. In order to obtain a greater variance, accepting that some less late seedlings are also preserved, a better strategy could be to set a threshold at a certain percentage. For example, the latest 25% of each population could be selected. Considering the fact that experience has shown that a large proportion of the offspring of cider apples will not have sufficient fruit quality, the selection should not be too sharp (personal communication). Until now it is not possible to make a statement based on the relation between the leaf budbreak time of the juvenile seedlings and the flowering time of the adult trees, as the trees did not flower yet. This has to be examined in the following years. A further issue that has to be focussed on in further investigations, is disease resistance. Because of the late flowering date, the infection process of different pathogen fungi is different from that of earlier flowering varieties. In the case of apple scab (*Venturia inaequalis*) it can be possible that because of the late start of the vegetation the initial ascospore infection is avoided. This could result in a lower infestation level through the whole vegetation period. Further, the presence of resistance markers as well an association study based on the current and future bud break data is planned and will aid future breeding for resistant late flowering apple varieties.

References

- Haug P., Rueß Dr. F., Zeiser A., Mayr U.M., Meyer M. Abschlussbericht zum Projekt "Robuste Apfelsorten für den Ökologischen Obstbau und den Streuobstbau". 2021.
- Legave, J. M., Guédon, Y., Malagi, G., el Yaacoubi, A., & Bonhomme, M. (2015). Differentiated responses of apple tree floral phenology to global warming in contrasting climatic regions. *Frontiers in Plant Science*, 6(DEC). <https://doi.org/10.3389/fpls.2015.01054>
- Miedaner T. (2010) Grundlagen der Pflanzenzüchtung. DLG Verlag
- Murawski H. (1959) Weitere Untersuchungen zur Züchtung von Apfelsorten mit spätem Laubaustrieb und Blühbeginn. *Beiträge zur Züchtungsforschung beim Apfel IV*. 29.2.
- Murawski H. (1967) Ergebnisse bei der Züchtung von Apfelsorten mit spätem Laubaustrieb und Blühbeginn. *Beiträge zur Züchtungsforschung beim Apfel IV*. 37.3.
- Rueß, F. (2020). Auswirkungen der Klimaverschiebung auf den Obstanbau und das Spätfrostisiko in der mittleren Neckarregion.
- Schmidt, M. (1940): Später Laubaustrieb und späte Blüte, ein Zuchtziel beim Apfel. *Der Züchter* 12, 281-289
- Snyder, R. L. & Melo-Abreu, J. P. de. (2005). Frost protection: fundamentals, practice and economics. Food and Agriculture Organization of the United Nations.
- Tydeman HM (1958) The breeding of late flowering apple varieties. Rpt. E- Malling Res. Sta. 1957:68-73.
- Mehlenbacher, S. A., & Voordeckers, A. M. (1991). Relationship of Flowering Time, Rate of Seed Germination, and Time of Leaf Budbreak and Usefulness in Selecting for Late-flowering Apples. In *J. AMER. Soc. HORT. SCI (Vol. 116, Issue 3)*.