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Original article

Exploring the role of tryptophan in biennial bearing: A mobile molecule that is exported from growing apples but does not suppress flowering

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Summary

Background of the study – **Apple (*Malus* × *domestica* Borkh.) production faces significant challenges, with biennial (or alternate) bearing being a major obstacle to stable yields. Natural high crop load (ON-year) considerably reduces return bloom in the following year resulting in low yields (OFF-year) that significantly diminish the profitability of apple production. Previous studies suggested that growing apples may export some unknown signaling molecule(s) to the adjacent bourse buds and thereby inhibit flower bud formation.** *Objectives* – **This study addresses the role of tryptophan as a potential signal that triggers biennial bearing. Previously, we found that bourse buds of high-cropping 'Fuji' and 'Gala' trees contain more tryptophan compared to bourse buds of the trees with no crop load. This led to the hypothesis that tryptophan is exported from the growing apples and accumulates in the adjacent bourse buds where it potentially inhibits flower initiation, or further metabolizes to melatonin, which may act as an inhibitor of flower bud formation.** *Methods* – **We tested our hypothesis by measuring the export of tryptophan from 'Fuji' and 'Gala' apples. Besides, we carried out foliar applications with tryptophan on 'Nicoter' and 'Gala' trees. In addition, we checked whether tryptophan or melatonin affect the continuous-flowering habit of RNAi transgenic apple plantlets with silenced expression of *MdTFL1* cultivated *in vitro*.** *Results* – **Our data shows that growing apple fruits of 'Fuji' and 'Gala' export tryptophan. However, foliar applications with this compound did not affect return bloom in 'Nicoter' or 'Gala'. Finally, neither tryptophan nor melatonin suppressed flowering of *MdTFL1-1* RNAi transgenic apple plantlets *in vitro*.** *Conclusions* – **Although the transport of tryptophan from apple fruits to adjacent bourse buds cannot be excluded, we reject the hypothesis on an inhibitory effect of tryptophan and its downstream metabolic product melatonin on flower bud formation in apple.**

Keywords
alternate bearing, apple diffusates, *Malus* × *domestica* Borkh., flower induction, fruit-bud interaction, melatonin

Significance of this study

What is already known on this subject?

- Biennial bearing, a common occurrence in apple orchards, entails irregular yields wherein seasons with high yields (ON-years) alternate with unproductive ones (OFF-years) if crop loads remain unadjusted. This cycle stems from a fruit-bud interaction, the physiological mechanism of which has not yet been elucidated. It is known that fruit growth overlaps with the formation of flower buds for the subsequent year. It is therefore hypothesized that apple fruit export some unknown mobile signals that disrupt the development of flower structures in adjacent bourse buds.

What are the new findings?

- The experiment with 'Fuji' and 'Gala' apple trees subjected to different crop load treatments revealed higher levels of tryptophan in bourse buds of high-cropping trees (ON-trees) compared to those without crop load (OFF-trees). Growing fruit of both cultivars export mobile tryptophan, suggesting that this compound could inhibit flower bud formation. However, foliar applications of tryptophan on 'Nicoter' and 'Gala' trees had no negative effect on return bloom. Furthermore, tissue culture experiments with continuously flowering *MdTFL1-1* RNAi plants showed no inhibitory effect of either tryptophan or melatonin treatments on the formation of flower meristems.

What is the expected impact on horticulture?

- This study provides insights for better understanding of biennial bearing in apple. Further research inspired by these findings could explore other potential regulatory molecules or mechanisms involved in flower bud initiation and biennial bearing. This work describes a method for tryptophan analysis in apple diffusates that may be of interest for further studies on fruit physiology.

Introduction

Apple is one of the most important fruits produced in the temperate zone. However, apple growers face many produc-

tion challenges that relate not only to environmental aspects but also to the biological characteristics of apple trees. One of the major constraints to obtaining stable apple yields is biennial (or alternate) bearing (Goldschmidt and Sadka, 2021). Biennial bearing is characterized by irregular yields of apple trees and often occurs if the crop load of the trees is not artificially adjusted by flower thinning or fruit thinning practices (Dennis, 2000). If the trees are left with their natural non-adjusted crop load, they produce a high number of small-sized fruit (ON-year) and the following year is typically unproductive (OFF-year) (Byers and Carbaugh, 2002; Meland, 2009). The probable reason for this phenomenon is that fruit growth overlaps with the formation of new flower buds, which burst and flower in the subsequent year (Monselise and Goldschmidt, 1982). It is therefore assumed that the normal formation of primordial flower structures in buds is inhibited by some unknown mobile signal(s) that apple fruit send to the adjacent bourse buds (Tromp, 2005). Many authors have shown that foliar applications of certain gibberellins reduce return bloom in apple (Elsysy and Hirst, 2019; McArtney, 1994). However, the mechanism behind this behavior remains unclear since evidence of the direct involvement of gibberellins in the inhibition of flower bud formation in apple is still scarce (Milyaev et al., 2022a). Interestingly, apple cultivars differ in their degree of biennial bearing. There are strongly biennial-bearing cultivars, such as 'Fuji', and more regular-bearing cultivars, such as 'Gala' (Hampson and Kemp, 2003). The origin of these differences is still largely unclear (Milyaev et al., 2022b).

Flower bud formation in fruit trees is commonly divided into three distinct stages. It starts with flower induction when the bud produces or perceives an unknown chemical signal for the formation of floral meristems (Kofler et al., 2019). There is evidence that flower induction in apple is regulated by the ratio of *FLOWERING LOCUS T (FT)* and the flowering repressor *TERMINAL FLOWER 1* (*TFL1*), which ultimately determines whether flower formation occurs or not, as was shown for *Arabidopsis thaliana* (Jaeger et al., 2013) and for apple (Flachowsky et al., 2012; Kotoda et al., 2010; Tränkner et al., 2010; Zuo et al., 2024). To date, there are no scientific methods that could be used to detect the stage of flower induction. The next stage of bud development is flower initiation, the time when the primordial flower tissues become detectable by bud histology and microscopy (Hanke, 1981). The last step that completes flower bud formation is flower differentiation, which occurs in the closed buds between late summer and early spring (Goeckeritz et al., 2023). In the current work, we will refer to "flower initiation" since this stage has been determined for 'Fuji' and 'Gala' in our previous studies (Kofler et al., 2019; Milyaev et al., 2018).

In our earlier work with 'Fuji' and 'Gala', the metabolic profile of apple bourse buds showed that the tryptophan concentration in buds was influenced by the crop load of the trees (Milyaev et al., 2021). In particular, we showed that the abundance of tryptophan in bourse buds collected from high-cropping trees (ON-trees) was higher than that in bourse buds from the trees with no crop load (OFF-trees). Moreover, we found that tryptophan was more abundant in bourse buds of the strongly biennial cultivar 'Fuji' compared to the bourse buds of the regular-bearing apple cultivar 'Gala'. This made tryptophan an interesting candidate to explore whether this compound or one of its metabolic products has an inhibitory effect on flower bud formation and whether the accumulation of tryptophan in bourse buds leads to biennial bearing in apple. One of the metabolic products of tryptophan is melatonin (Arnao and Hernández-Ruiz, 2020). In the model plant Arabidopsis, this compound has been shown to stabilize DELLA proteins and to inhibit the floral transition (Shi et al., 2016). DELLA proteins are inhibitors of gibberellin signaling, act as growth repressors (Hedden, 2020), and could potentially link tryptophan with the effect of gibberellins on flower initiation in apple. In Arabidopsis, DELLA proteins suppress the expression of *FT*, an integrator gene of flower induction (Takagi et al., 2023).

One of the limitations of the study of Milyaev et al. (2021) was that the metabolites including tryptophan were analyzed in single buds, resulting in considerable variation of tryptophan concentrations across replicates. In the current work, we aimed to validate the previous results by analyzing tryptophan in pooled samples of 20 bourse buds per replicate, which originated from the same experiment with 'Fuji' and 'Gala' (Milyaev et al., 2021). In this study we hypothesized that export of tryptophan from growing apples could explain its accumulation in the bourse buds of high-cropping apple trees. Besides, we examined the possibility that tryptophan inhibits flower bud formation in apple. Therefore, the objectives of the current work were (1) to measure the export of tryptophan from the growing apple fruits of 'Fuji' and 'Gala'; (2) to compare tryptophan content in apple seeds and in apple fruit flesh of both apple cultivars to identify the locations within fruits that have particularly high tryptophan concentrations; (3) to study the effect of foliar applications of tryptophan on return bloom of 'Nicoter' apple trees and the effect of tryptophan and its potential conversion product melatonin on return bloom of 'Gala' trees; (4) to test the effect of tryptophan and melatonin on transgenic *MdTFL1-1* RNAi apple plantlets in tissue culture. In these plants, the *TFL1* gene of apple *MdTFL1* is silenced, shifting the ratio between MdTFL1 and MdFT in favor of MdFT which results in continuous flowering (Flachowsky et al., 2012). If tryptophan or its downstream metabolite melatonin prevent the expression of *MdFT* by, e.g., stabilizing the DELLA proteins, flower formation in the transgenic plants should be inhibited.

Materials and methods

Experimental orchard and sampling material for the analysis of tryptophan

For a detailed description of the experimental design and sampling, the reader is referred to Milyaev et al. (2021). In brief, the experimental apple orchard was located at the Center of Competence for Fruit Cultivation (KOB) at Lake Constance near Ravensburg, Germany (47°46'2.89"N, 9°33'21.21"E, alt. 490 m). The field experiment was conducted with 9-year-old apple trees of 'Royal Gala' (further 'Gala') and 'Fuji', which were grafted on M.9 rootstock. These trees were planted at a density of 3.0 × 1.0 m, pruned and trained as tall spindles (4.5 m height) and grown according to the common management practices for high-density orchards recommended for the region. In 2015 we applied two contrasting crop load treatments to each of the cultivars: heavy (natural) crop load (ON-trees) and no crop load, which was achieved by removing all flowers at full bloom (OFF-trees). For the analysis of metabolites (including tryptophan), bourse buds were collected at weekly intervals during the period of flower bud development (28–125 days after full bloom, DAFB). Bourse buds were collected as pooled samples of 20 bourse buds from each tree in 4 biological replicates (tree = biological replicate). The buds were cut from the trees, placed in 2 mL safe-lock Eppendorf tubes (Eppendorf

AG, Hamburg, Germany), snap-frozen in liquid nitrogen and stored at -80°C until they were analyzed. Histological analyses that were a part of the same study showed that flower initiation in 'Fuji' and 'Gala' occurred at 75 DAFB and at 97 DAFB, respectively (Kofler et al., 2019; Milyaev et al., 2022a). To confirm the results of our previous study that the relative abundance of tryptophan in bourse buds collected from ON-trees is higher compared to bourse buds sampled from OFF-trees (Milyaev et al., 2021), we took bourse buds from the same sample set and re-analyzed those belonging to the two selected sampling time points: 68 DAFB for 'Fuji' and 89 DAFB for 'Gala'. These sampling dates preceded the detected flower initiation for 'Fuji' and 'Gala', respectively. More data on relative abundance of tryptophan in bourse buds of 'Fuji' (48–68 DAFB) and 'Gala' (68–89 DAFB) is published in Milyaev et al. (2021).

Tryptophan export from apple fruit was measured using apple fruit diffusates. The sampling procedure and the diffusate preparation are comprehensively described in Milyaev et al. (2022a). In short, apple fruit were harvested from the same experimental orchard with commercial (moderate) crop load in 2020 at 55, 68, 83, and 97 DAFB. From four randomly selected apple trees (per cultivar, tree = biological replicate), we harvested eight apples at each sampling time point. Harvested apples were immediately immersed with their pedicels in 2.5 mL of phosphate buffer solution (0.1 M phosphate buffer at pH of 6.2 containing 20 mM ethylenediaminetetraacetate, EDTA), transferred to the lab and incubated at 20°C for 20 h. After incubation, the apples were discarded, and the eight tryptophan-containing buffer solutions belonging to the same replicate were pooled together, frozen and stored at -20°C until analyzed. At 97 DAFB, at the time when apple seeds were partially brown, we sampled an additional fruit set for the analysis of tryptophan in seeds and fruit flesh. To this end, four fruit per tree were sampled from 'Fuji' and 'Gala' in four biological replicates (tree = biological replicate). Apples were taken to the lab, where the seeds of four fruit belonging to the same replicate were placed in 2 mL safe-lock Eppendorf tubes and snap-frozen in liquid nitrogen. Fruit flesh samples of approximately 2 cm^3 per fruit were taken from the fruit cortex close to the fruit core. The samples belonging to the same replicate were placed in 15 mL Eppendorf falcon tubes and snap-frozen in liquid nitrogen. Both tissues were stored at -80°C until required.

Sample preparation

The sample preparation has been described in detail in Milyaev et al. (2022a). In brief, apple buds, seeds, and fruit flesh were ground to powder using a cryogenic mixer mill (CryoMill, Retsch GmbH, Haan, Germany). For tryptophan analysis, we used 30 ± 5 mg fresh weight (FW) of the frozen plant tissue. Tryptophan was extracted using the protocol of Šimura et al. (2018) with the modifications published in Milyaev et al. (2022a). At the final step, the extracts were dissolved in 50 µL of 30% acetonitrile containing 0.1% formic acid and transferred to insert-equipped vials. Apple diffusates (50 µL of each replicate) were transferred to insert-equipped vials without any additional sample preparation.

Analysis of tryptophan

Analysis of tryptophan in apple buds, seeds, and fruit flesh was performed using UPLC-MS/MS as described in Eggert and von Wirén (2017). This method aimed at relative quantification of tryptophan and was therefore applied without using an internal, isotope-labeled tryptophan standard. This allowed statistical comparisons between contrasting treatments and tissues (e.g., ON vs. OFF, 'Fuji' ON vs. 'Gala' ON, or seeds vs. fruit flesh), but did not allow determining absolute concentrations of tryptophan in buds, seeds, and fruit flesh. The presence of tryptophan in these samples was later confirmed by a spiking test with the L-tryptophan standard (purity ≥ 99.5%) and one of the samples. The tryptophan standard was purchased from Merck KGaA (Darmstadt, Germany).

For the analysis of tryptophan in apple diffusates, we used an HPLC-MS/MS system consisting of an HPLC Agilent 1290 (Santa Clara, CA, U.S.A.) coupled with the AB Sciex QTRAP 5500 mass-spectrometer (AB Sciex LLC, Framingham, MA, U.S.A.). The HPLC unit was equipped with a Supelco column, type Discovery® HS C18, L × I.D. 15 cm × 2.1 mm, 3 µm particle size. The column temperature was set to 40.0°C. The sample injection volume was 5 µL. As a mobile phase, we used 0.2% formic acid (solvent A) and acetonitrile containing 0.2% formic acid (solvent B). The flow of the mobile phase was 0.4 mL min^{-1} at the following gradient: min 0.0–3.9 – 100% A (discarded fraction), min 4.0–6.4 – 100% A (this and the following fractions were directed to the MS), min 6.5–7.9 – 30% A and 70% B, min 8.0–10.9 – 100% B, min 11.0–11.9 – 100% B. Tryptophan was measured in the positive ionization mode after setting the mass-spectrometer to a capillary voltage of 5.5 kV, a desolvation temperature of 550°C, curtain gas (N_2) – 35 psi (pounds per square inch), collision gas (N_2) – medium, gas 1 (air) – 30 psi, gas 2 (air) – 60 psi. Ions were monitored in Multiple Reaction Monitoring (MRM) mode. Specifically, for ion transition from tryptophan parent ion (205.0 Da) to the fragment ion of 188.0 Da, the following parameters were applied: collision energy (CE) of 12 V, declustering potential (DP) of 75 V, entrance potential (EP) of 10 V, collision cell exit potential (CXP) of 12 V. For ion transition from 205.0 Da to 146.0 Da, all the parameters were kept identical except for CE, which was set to 17 V. This method allowed absolute tryptophan quantification in the diffusates.

Foliar applications of tryptophan in apple orchards

A field experiment with 12-year-old trees of the 'Nicoter' (Kanzi®) apple cultivar was carried out in the experimental orchard of the University of Hohenheim (48°42'44.3"N, 9°11'33.5"E), Stuttgart, Germany. The trees were grafted on M.9 rootstock, planted at 3.0 × 1.0 m spacing, pruned as thin spindles and trained according to the common local practices for high-density orchards. In 2021, we randomly selected 10 experimental trees that grew in two adjacent rows. All the flowers on these trees were removed at full bloom. The trees were randomly allocated to two groups: tryptophan application (5 trees) and control treatment with water (5 trees). Prior to foliar applications, tryptophan was diluted in tap water to a final concentration of 8 g L^{-1} (close to the tryptophan-saturated solution). To each tryptophan solution, we added 0.5 mL L^{-1} of the surfactant Break-Thru S 301 (Alzchem Group AG, Trostberg, Germany). L-tryptophan (purity ≥ 99.8%) was obtained from Sunday Natural Products GmbH (Berlin, Germany). The trees were sprayed to run-off 11 times at weekly intervals + one additional treatment at 107 DAFB. Treatments were applied on the following DAFB: 23, 30, 37, 44, 52, 58, 65, 71, 78, 85, 91, and 107. Control 'Nicoter' trees were sprayed with water + 0.5 mL L^{-1} Break-Thru S 301 on the same days. In the following spring, the trees were not pruned; all spur buds and all flower clusters (not only those of spur buds) were counted on each tree.

Foliar applications of tryptophan and melatonin were performed in an orchard of the Julius Kühn Institute in

Dresden-Pillnitz (51°00'00.8"N, 13°53'13.2"E). For the experiment, we used 11-year-old 'Gala' trees grafted on M.9 rootstock with commercial (moderate) crop load. Each compound was applied on 15 randomly selected spurs (leaves and bourse buds) per replicate in three biological replicates (tree = biological replicate). The treatments were carried out systematically on the same fruit-bearing spurs. The melatonin working solution contained 250 µM (58.07 mg L^{-1}) melatonin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany; purity > 97%), 1.25 mL L^{-1} ethanol, and 0.01% v/v Tween® 20 (Merck KGaA, Darmstadt, Germany). The control water solution contained the equivalent amounts of Tween® 20 and ethanol, which were used to prepare the melatonin working solution. Tryptophan solution was prepared by diluting 1 g of L-tryptophan (Vita-World GmbH, Taunusstein, Germany; purity 82%) in 1 L water and adding 0.01% v/v Tween® 20. All the selected 'Gala' spurs (in total 45 spurs per treatment) were sprayed to run-off five times at weekly intervals (23 June, 1 July, 7 July, 15 July, and 22 July 2021). In the following spring, return bloom was scored on every surviving spur.

Tissue culture experiments with tryptophan and melatonin

To test whether tryptophan or melatonin can inhibit the induction of floral meristems, the continuously flowering transgenic *MdTFL1-1* RNAi lines M0773 and M0777 of the apple genotype 'PinS' (Flachowsky et al., 2012) were treated with melatonin and tryptophan. These transgenic lines were replicated and cultivated on a standard Murashige and Skoog (MS) medium (8 g L^{-1} agar, 30 g L^{-1} sorbitol, 1 mg mL^{-1} BAP, 1 mg mL^{-1} IBA, pH = 5.8). For treatments, plants were transferred either to the standard MS medium (control for tryptophan), MS medium with ethanol (control for melatonin), MS medium with 500 µM tryptophan (102.11 mg L^{-1}) or MS medium with 500 µM melatonin (116.14 mg L^{-1}). For each treatment, 30–36 plantlets (*in vitro* explants) were cultivated in three glasses. All main and side shoots of each explant (= biological replicate) were scored for the presence of vegetative shoot tip or inflorescence by visual inspection six weeks after the explants had been transferred to the corresponding media. The data allowed presenting the number of shoots (vegetative + flowering shoots) per explant and the total share of flowering shoots per treatment (all explants).

Statistical tests and illustrations

Student's *t*-test (with a significance level of $p < 0.01$) was performed using SigmaPlot 14.0 (Systat Software GmbH). All figures that are shown in the current work were created using the same software. The Wilcoxon tests for the number of shoots per explant and for the percentages of flowering shoots *in vitro* was done in R with the package ggpubr (Kassambara, 2023).

Results

In order to establish a possible link between crop load of the trees and tryptophan concentration in bourse buds, it was necessary to confirm the previous result that the abundance of tryptophan is indeed higher in bourse buds of high-cropping trees, especially during the critical period of flower initiation. We therefore analyzed bourse buds of 'Fuji' and 'Gala' with contrasting crop load treatments (ON versus OFF) at the sampling time points that preceded the onset of flower initiation for both apple cultivars. Since flower initiation occurred 75 DAFB for 'Fuji' and 97 DAFB for 'Gala', we chose the following sampling time points to determine the differences between ON- and OFF-treatments: 68 and 89 DAFB for 'Fuji' and 'Gala', respectively. We found the abundance of tryptophan at 68 DAFB in bourse buds of 'Fuji' ON to be 4.7-fold higher compared to bourse buds of 'Fuji' OFF. For 'Gala' at 89 DAFB, the difference between ON- and OFF-treatments was 2.9-fold with higher tryptophan concentration in bourse

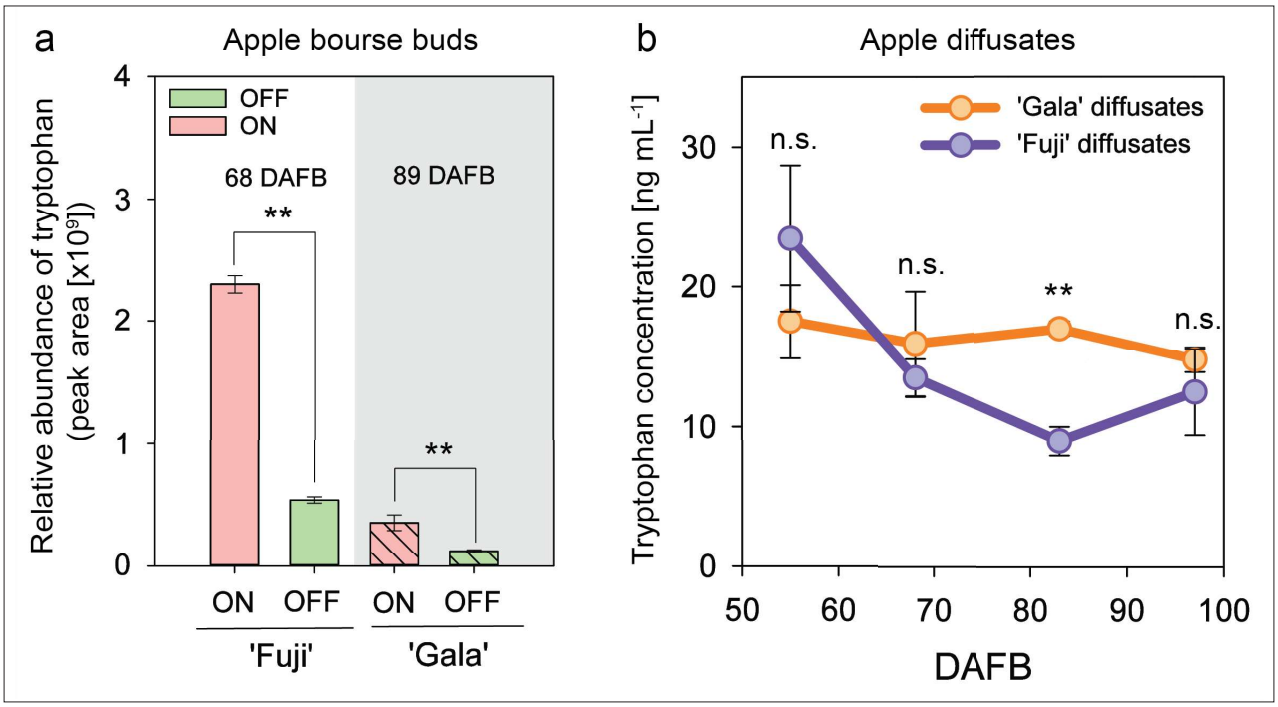


Figure 1. Relative abundance of tryptophan in bourse buds of 'Fuji' at 68 DAFB and 'Gala' at 89 DAFB (a) and tryptophan concentration in apple fruit diffusates of 'Fuji' and 'Gala' at 55, 68, 83, and 97 DAFB (b). N.s. indicates no significant differences whereas two asterisks indicate significant differences at $p < 0.01$. Error bars represent standard deviation. Sample size was four replicates, each of them consisting of either 20 bourse buds (a) or eight apple diffusates (b).

buds collected from ON-trees (Figure 1a). Both differences were statistically significant.

Based on our finding that high crop load led to the accumulation of tryptophan in apple bourse buds, the next step was to investigate whether apple fruit are able to export mobile tryptophan. We used diffusates of apple fruit harvested at 55, 68, 83, and 97 DAFB to measure mobile tryptophan in the diffusate buffer solution. The result clearly showed that tryptophan was exported from apple fruits of both 'Fuji' and 'Gala'. However, despite a statistical difference between tryptophan concentrations of 'Fuji' and 'Gala' diffusates at 83 DAFB, there were no clear differences in tryptophan export across studied cultivars (Figure 1b).

The next question that we aimed to answer was whether seeds or fruit flesh tissues are the main tryptophan sources in apple fruit. The results showed that at 97 DAFB tryptophan concentration in apple seeds was 15.7-fold and 11.2-fold higher than in fruit flesh of 'Fuji' and 'Gala', respectively (Figure 2). Moreover, we also observed cultivar differences: 'Fuji' seeds contained 5.1 times more tryptophan than 'Gala' seeds, whereas 'Fuji' fruit flesh had 3.7-fold more tryptophan compared to fruit flesh of 'Gala' apples.

The current work also addressed the question whether tryptophan suppresses return bloom when sprayed in the orchard during the time of flower bud formation. For this purpose, we chose the biennial-bearing cultivar 'Nicoter' and the regular-bearing cultivar 'Gala'. The results showed that foliar applications of tryptophan (12 times at 8 g L^{-1}) affected neither the number of spurs (Figure 3a), nor the number of flower clusters (return bloom) on 'Nicoter' trees (Figure 3b). The targeted treatments of 'Gala' spurs (5 times) with either tryptophan (at 1 g L^{-1}) or melatonin (at 250 µM, or 58.07 mg L^{-1}) also showed no effect on return bloom in the following year (Figure 4).

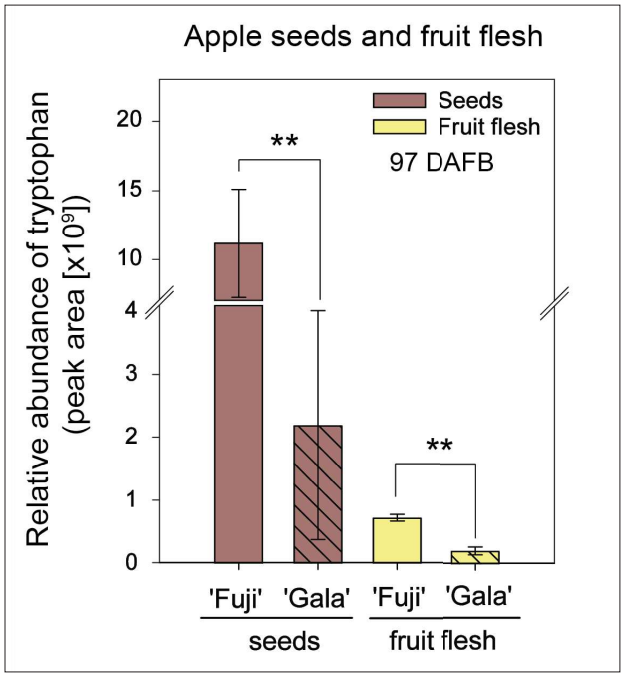


Figure 2. Relative abundance of tryptophan in apple seeds and fruit flesh of 'Fuji' and 'Gala' at 97 DAFB. Two asterisks indicate significant differences at $p < 0.01$. Error bars represent standard deviation. Sample size was four replicates, each of them consisting of seeds or fruit flesh taken from four apples.

Finally, we tested the effect of tryptophan and melatonin on flower initiation of the *in vitro* cultivated *MdTFL1-1* RNAi lines M0773 and M0777. These transgenic lines lost the ability to inhibit flowering and hence flower continuously. Newly

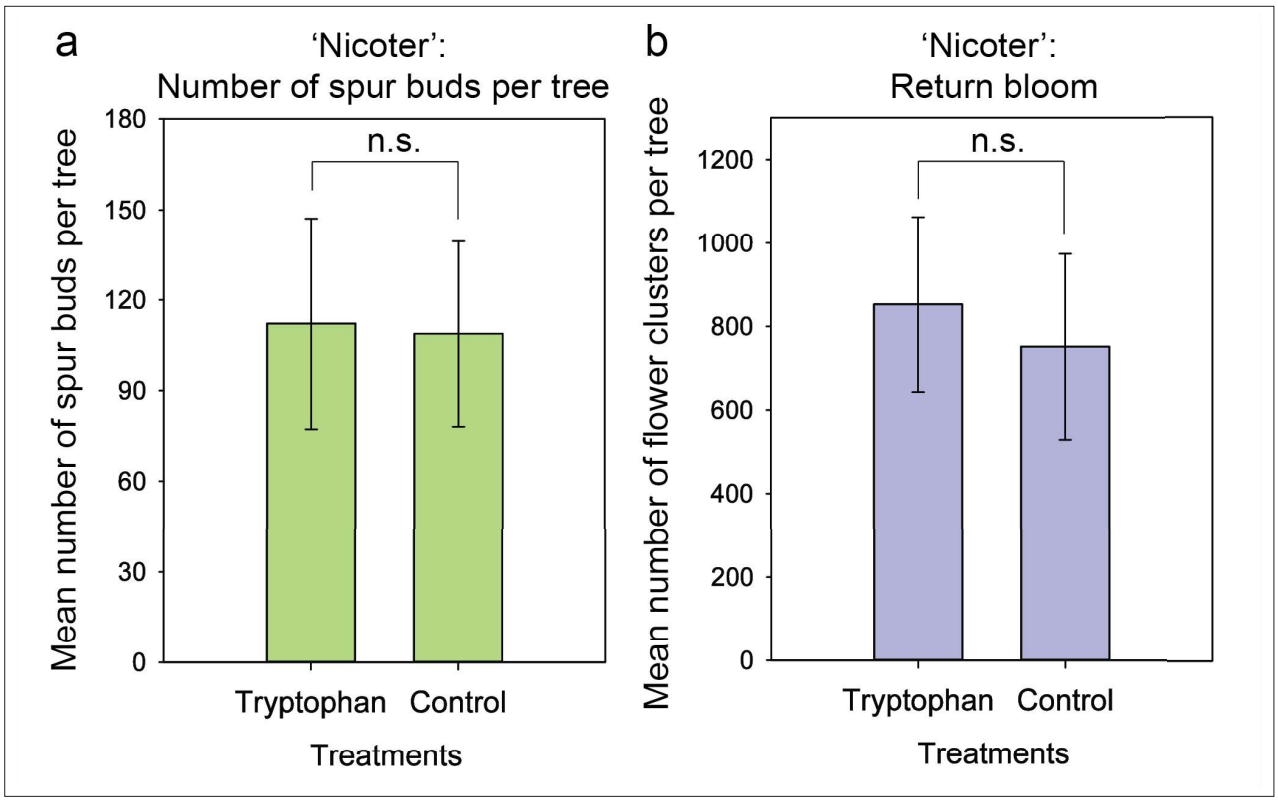


Figure 3. Number of spurs (a) and number of flower clusters (return bloom, b) on 'Nicoter' trees after 12 tryptophan applications (8 g L^{-1}) compared to the control (trees sprayed with water). N.s. indicates no significant differences at $p < 0.01$. Error bars represent standard deviation. Sample size was five 'Nicoter' trees per treatment.

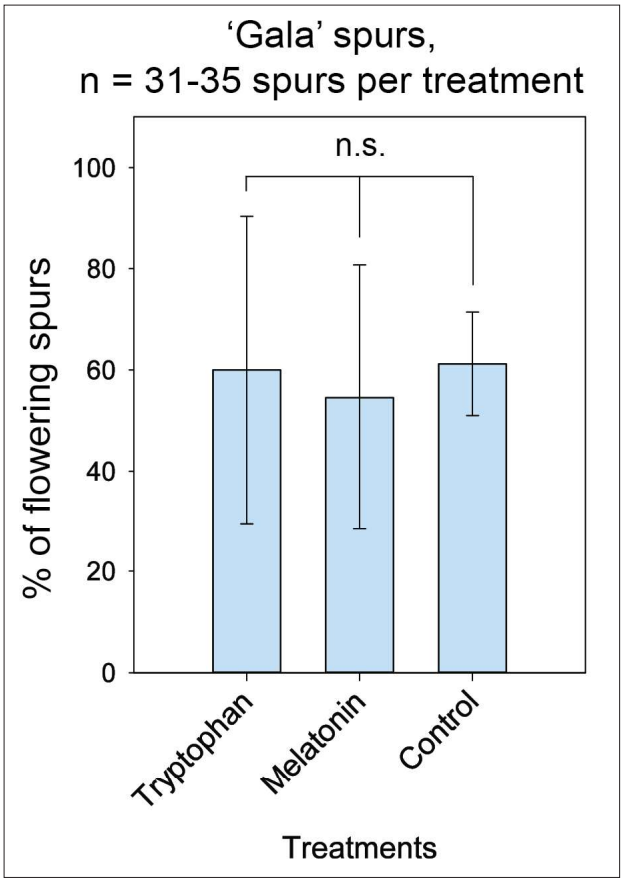


Figure 4. Percentage of flowering spurs on 'Gala' trees after 5 applications with either melatonin (58.07 mg L^{-1}) or tryptophan (1 g L^{-1}) compared to the control (trees sprayed with water). N.s. indicates no significant differences at $p < 0.01$. Error bars represent standard deviation. Sample size was 31–35 spurs per treatment.

formed meristems develop either into side shoots or into flower structures. Therefore, these lines provide a good experimental system to test the effect of tryptophan and melatonin on flower initiation. On average, 3.0 to 4.1 shoots per explant developed after the start of the treatment until the scoring day (day 42, Figure 5a, b). The percentage of shoots that transitioned to flowers did not differ significantly between treatments and controls for both tryptophan and melatonin, as well as for both transgenic *MdTFL1-1* RNAi lines tested (Figure 5c, d).

Discussion

The physiological mechanism that apple fruits employ to inhibit flower initiation in the adjacent bourse buds has not yet been elucidated (Goldschmidt and Sadka, 2021). To explore this fruit-bud interaction, we analyzed metabolic profiles of apple bourse buds from 'Fuji' and 'Gala' with high and zero crop loads (Milyaev et al., 2021). We demonstrated that bourse buds that grow and develop next to apple fruit respond to the presence of the fruit with increased tryptophan concentrations compared to the buds of the trees with no crop load. Moreover, tryptophan was more abundant in bourse buds of the strongly biennial apple cultivar 'Fuji' compared to the regular-bearing cultivar 'Gala' (Figure 1a). This abundance pattern of tryptophan in bourse buds suggested that this amino acid may act as an inhibitor of flower initiation in apple. Another strong reason to focus on tryptophan in the current work is that this compound is apparently exported from apple fruit (Figure 1b) and could therefore be transported to the adjacent bourse buds. To our current knowledge, the scientific literature does not provide any information on tryptophan in connection to flower induction of perennial plants. However, tryptophan is known to serve as a direct precursor of several plant growth-regulating compounds, such as auxin indole-3-acetic acid (IAA) (Enders and Strader, 2015) and melatonin (Murch and Erland, 2021).

In works with other biennial-bearing fruit tree species, the presence of fruits affected the concentrations of IAA conjugates in apical buds in avocado (Pochamreddy et al., 2024) and increased IAA polar transport in shoots in citrus and olive (Haim et al., 2021). However, in our study with 'Fuji' and 'Gala' apple trees, targeted analysis of IAA in bourse buds failed to detect IAA in 'Fuji' whereas the IAA abundance in 'Gala' did not differ significantly between bourse buds collected from ON- and OFF- trees in the period of 68–118 DAFB (Milyaev et al., 2022a). Therefore, the differences of tryptophan abundances between ON- and OFF-treatments in 'Gala' do not seem to stem from different intensities of tryptophan conversion into IAA. To check the abundance of melatonin in the selected bourse bud samples, we analyzed the same bud extracts that we prepared for the analysis of tryptophan ('Fuji' – 68 DAFB and 'Gala' – 89 DAFB, Figure 1a). The result showed no differences in melatonin concentrations between the ON- and OFF-treatments for both studied apple cultivars (data not shown).

To test whether tryptophan inhibits flower bud formation in apple, we prepared tryptophan-water solutions (8 g L^{-1}) and sprayed 'Nicoter' trees during the growing season. In the following spring, the trees flowered as usual and return bloom was not affected by the high concentrations of applied tryptophan (Figure 3b). By the end of the growing season, all treated trees looked healthy, the shoot growth and other canopy parameters of the treated trees did not seem to differ from the untreated ones (visually estimated). However, the elasticity of leaves on the tryptophan-treated trees decreased and the leaves became brittle. In previous studies, foliar applications of tryptophan in apple orchards were assessed for effects on fruit quality and yield. For example, Mosa et al. (2021) sprayed 'Anna' apple trees with 25–100 mg L^{-1} tryptophan before flowering, at full bloom, and one month after full bloom. The treatments reduced fruit drop and positively affected fruit set, yield, and fruit quality. Besides, the yield of control and tryptophan-treated trees was stable in both experimental years (2018 and 2019). This indirectly indicates that flower bud formation was not affected by tryptophan treatments. Similarly, no yield reduction was reported for the biennial-bearing apple cultivar 'Elstar' after it was sprayed with tryptophan (100 mg L^{-1}) six times between budbreak and 28 DAFB in 2014–2016 (Wójcik et al., 2019) and for 'Le-Conte' pear trees sprayed with 50 and 100 mg L^{-1} of tryptophan at full bloom as well as three weeks later in 2015 and 2016 (Khedr, 2018). In the abovementioned study with 'Elstar' (Wójcik et al., 2019) and in an identical field trial with 'Red Jonaprince' apple trees (Wójcik et al., 2016), tryptophan treatments increased the concentration of fruit calcium in both apple cultivars and IAA concentration in 'Red Jonaprince' fruitlets at 42 days after petal fall (the IAA concentration in 'Elstar' fruitlets was not measured). There is generally little knowledge on the extent to which leaves absorb amino acids after foliar applications (Teixeira et al., 2018). However, the effects of tryptophan applications on fruit quality and yield in apple (discussed above) indicate that at least some amount of tryptophan can be taken up by apple leaves. The question of

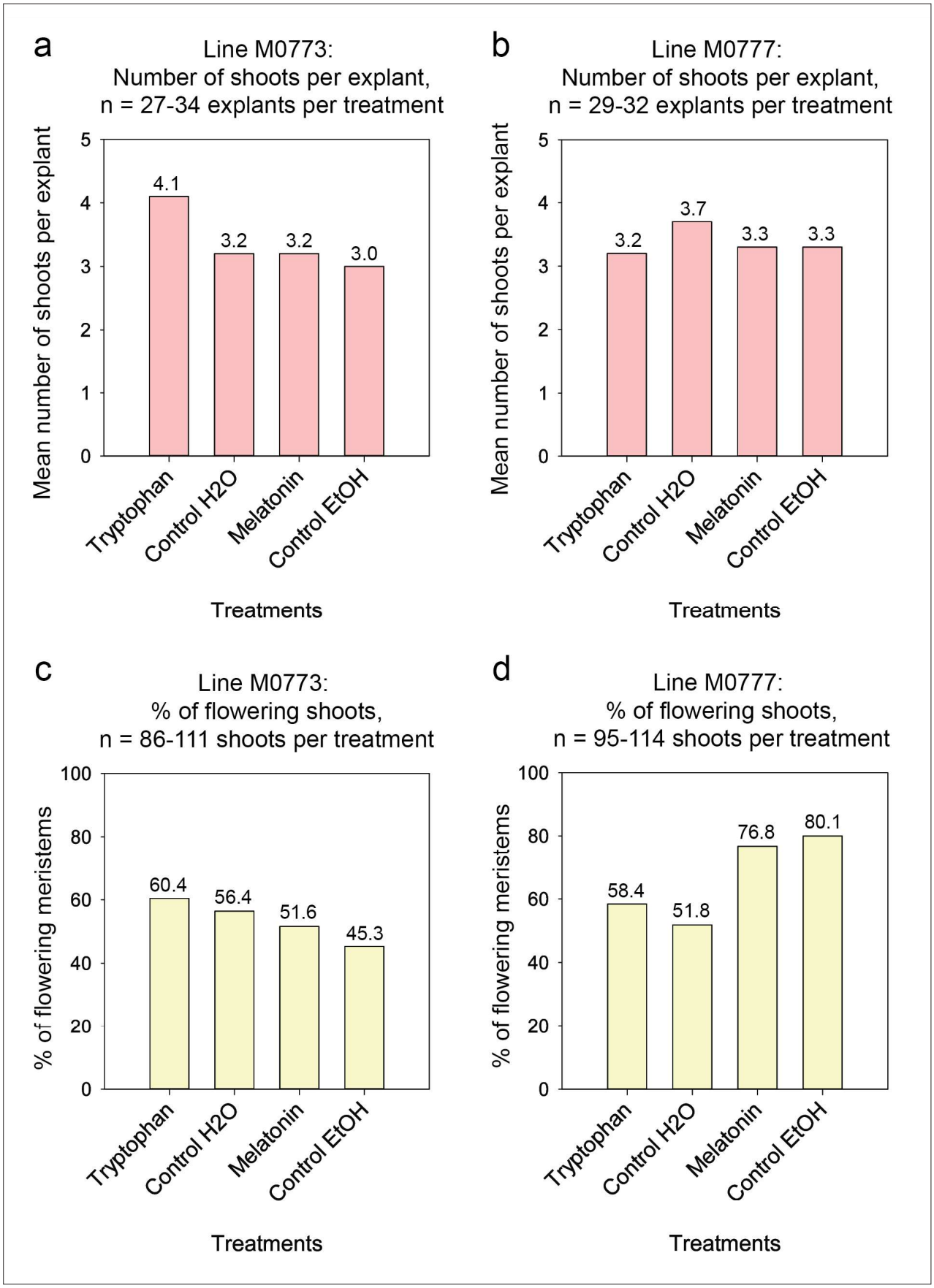


Figure 5. Number of shoots per explant (a, b) and percentage of flowering shoots (c, d) of the *MdTLF1-1* RNAi lines M0773 (a, c) and M0777 (b, d) cultivated *in vitro* on MS media containing either tryptophan (500 µM) or melatonin (500 µM). Tryptophan treatments were compared to the "control H_2O" whereas the treatments with melatonin were compared to the "control EtOH" (ethanol) as melatonin was diluted in ethanol prior to its application. Tryptophan and melatonin did not show any significant effects on the displayed parameters. Sample size (*n*) is shown above each figure.

how much tryptophan ultimately reaches the buds after being sprayed on the leaves remains open for further research.

In plants, tryptophan also serves as a precursor of another signaling molecule – melatonin (Arnao and Hernández-Ruiz, 2020), which appears to be involved in the control of stress responses, growth and senescence and has been implicated in the control of flowering in *A. thaliana* (Arnao and Hernández-Ruiz, 2020; Shi et al., 2016). Melatonin has been described as stabilizing DELLA proteins, which are repressors of gibberellin signaling (Arnao and Hernández-Ruiz, 2020). For this reason, not only tryptophan but also melatonin was tested in the experiments with 'Gala' trees in regard to flower bud formation. Targeted applications of these compounds on spurs, however, did not affect return bloom in 'Gala' (Figure 4). Therefore, these experiments did not support our hypothesis that tryptophan or melatonin inhibit flower induction in apple. Further evidence that led to the rejection of this hypothesis came from the *in vitro* experiments with the transgenic *MdTFL1-1* RNAi apple lines, which continuously form floral meristems. If tryptophan or melatonin were able to inhibit flower induction in *MdTFL1-1* RNAi apple lines, we would expect a significantly reduced number of flowers in these lines when treated with either of the two compounds. However, neither tryptophan nor melatonin significantly altered the percentages of flowering shoots that the *in vitro* explants of the transgenic lines formed within 42 days since the beginning of the corresponding treatments (Figure 5c, d).

The mobile signal that apple fruits send to the adjacent bourse buds to inhibit flower bud initiation has not yet been discovered. It is still widely assumed that the potential inhibitor of flower initiation belongs to the class of gibberellins (Elsysy and Hirst, 2019; McArtney, 1994). The challenging task for further studies will be to explore the diversity of gibberellins and other molecules that can be transported from apple fruit to the neighboring organs, such as bourse buds, to suppress flower initiation. In addition, new and advanced tools for untargeted metabolomics and proteomics might also shed some light on the physiological interaction between fruits and buds, especially in relation to flower bud development.

Conclusion

The experimental setup with 'Fuji' and 'Gala' using contrasting crop load treatments showed higher abundance of tryptophan in bourse buds of high-cropping trees (ON-trees) compared to trees without crop load (OFF-trees). Bourse buds of the biennial-bearing cultivar 'Fuji' contained significantly more tryptophan than the bourse buds of the regular-bearing cultivar 'Gala'. We showed that tryptophan is a mobile compound that was exported from the growing apples of both cultivars. Tryptophan concentrations in apple seeds were significantly higher than those in fruit flesh. All these findings made tryptophan an interesting candidate that could inhibit flower bud initiation in apple. However, foliar applications of 'Nicoter' trees with tryptophan affected neither the number of spurs nor return bloom and hence failed to suppress flower bud formation in 'Nicoter'. A similar experiment with 'Gala' confirmed these findings. In addition, foliar applications of melatonin, a downstream metabolite of tryptophan, also did not influence return bloom in 'Gala'. These findings were in line with our tissue culture experiments, in which we used continuously flowering transgenic *MdTFL1-1*-silenced apple lines grown on culture media containing either tryptophan or melatonin. In conclusion, tryptophan and melatonin are probably not the molecules that inhibit flower initiation in apple.

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References

Arnao, M.B., and Hernández-Ruiz, J. (2020). Melatonin in flowering, fruit set and fruit ripening. Plant Reprod. *33*, 77–87. https://doi.org/10.1007/s00497-020-00388-8.

Byers, R.E., and Carbaugh, D.H. (2002). Effects of thinning time on yield, fruit size, and return bloom of 'York' and 'Golden Delicious' apple trees. J. Tree Fruit Prod. *3*, 55–62. https://doi.org/10.1300/J072v03n01_05.

Dennis, F.G. (2000). The history of fruit thinning. Plant Growth Regul. *31*, 1–16. https://doi.org/10.1023/A:1006330009160.

Eggert, K., and Von Wirén, N. (2017). Response of the plant hormone network to boron deficiency. New Phytol. *216*, 868–881. https://doi.org/10.1111/nph.14731.

Elsysy, M.A., and Hirst, P.M. (2019). Molecular basis of flower formation in apple caused by defoliation and gibberellins. J. Am. Soc. Hortic. Sci. *144*, 414–419. https://doi.org/10.21273/JASHS04760-19.

Enders, T.A., and Strader, L.C. (2015). Auxin activity: Past, present, and future. Am. J. Bot. *102*, 180–196. https://doi.org/10.3732/ajb.1400285.

Flachowsky, H., Szankowski, I., Waidmann, S., Peil, A., Trankner, C., and Hanke, M.-V. (2012). The *MdTFL1* gene of apple (*Malus* × *domestica* Borkh.) reduces vegetative growth and generation time. Tree Physiol. *32*, 1288–1301. https://doi.org/10.1093/treephys/tps080.

Goeckeritz, C.Z., Gottschalk, C., Van Nocker, S., and Hollender, C.A. (2023). Malus species with diverse bloom times exhibit variable rates of floral development. J. Am. Soc. Hortic. Sci. *148*, 64–73. https://doi.org/10.21273/JASHS05236-22.

Goldschmidt, E.E., and Sadka, A. (2021). Yield alternation: Horticulture, physiology, molecular biology, and evolution. In Horticultural Reviews, Vol. 48, I. Warrington, ed. (Wiley), p. 363–418. https://doi.org/10.1002/9781119750802.ch8.

Haim, D., Shalom, L., Simhon, Y., Shlizerman, L., Kamara, I., Morozov, M., Albacete, A., Rivero, R.M., and Sadka, A. (2021). Alternate bearing in fruit trees: Fruit presence induces polar auxin transport in citrus and olive stem and represses IAA release from the bud. J. Exp. Bot. *72*, 2450–2462. https://doi.org/10.1093/jxb/eraa590.

Hampson, C.R., and Kemp, H. (2003). Characteristics of important commercial apple cultivars. In Apples: Botany, Production and Uses (Guildford and King's Lynn, U.K.: Biddles Ltd.), p. 61–89.

Hanke, M.-V. (1981). Histologische Untersuchungen zur Blütenknospendifferenzierung bei *Malus domestica* Borkh. (Dresden, Germany: Akademie der Landwirtschaftswissenschaften DDR).

Hedden, P. (2020). The current status of research on gibberellin biosynthesis. Plant Cell Physiol. *61*, 1832–1849. https://doi.org/10.1093/pcp/pcaa092.

Jaeger, K.E., Pullen, N., Lamzin, S., Morris, R.J., and Wigge, P.A. (2013). Interlocking feedback loops govern the dynamic behavior of the floral transition in *Arabidopsis*. Plant Cell *25*, 820–833. https://doi.org/10.1105/tpc.113.109355.

Kassambara, A. (2023). ggpubr: 'ggplot2' Based Publication Ready Plots. R package v. 0.6.0 (WWW Document). R Packages. https://rpkgs.datanovia.com/ggpubr/ (accessed September 2, 2024).

Khedr, E. (2018). Improving productivity, quality and antioxidant capacity of Le-Conte pear fruits using foliar tryptophan, arginine and salicylic applications. Egypt. J. Hortic. *45*, 93–103. https://doi.org/10.21608/ejoh.2018.3313.1057.

Kofler, J., Milyaev, A., Capezzone, F., Stojnić, S., Mićić, N., Flachowsky, H., Hanke, M.-V., and Wünsche, J.-N. (2019). High crop load and low temperature delay the onset of bud initiation in apple. Sci. Rep. *9*, 1–11. https://doi.org/10.1038/s41598-019-54381-x.

Kotoda, N., Hayashi, H., Suzuki, M., Igarashi, M., Hatsuyama, Y., Kidou, S., Igasaki, T., Nishiguchi, M., Yano, K., Shimizu, T., Takahashi, S., Iwanami, H., Moriya, S., and Abe, K. (2010). Molecular characterization of *FLOWERING LOCUS T*-Like genes of Apple (*Malus* × *domestica* Borkh.). PCP *51*, 561–575. https://doi.org/10.1093/pcp/pcq021.

McArtney, S.J. (1994). Exogenous gibberellin affects biennial bearing and the fruit shape of 'Braeburn' apple. N. Z. J. Crop Hortic. Sci. *22*, 343–346. https://doi.org/10.1080/01140671.1994.9513844.

Meland, M. (2009). Effects of different crop loads and thinning times on yield, fruit quality, and return bloom in *Malus* × *domestica* Borkh. 'Elstar.' J. Hortic. Sci. Biotechnol. *84*, 117–121. https://doi.org/10.1080/14620316.2009.11512607.

Milyaev, A., Kofler, J., Pfannstiel, J., Stefanelli, D., Flachowsky, H., Hanke, M.V., and Wünsche, J.N. (2018). Histological and proteomic approaches to study floral bud induction in relation to biennial bearing in apple. Acta Hortic. *1229*, 277–284. https://doi.org/10.17660/ActaHortic.2018.1229.42.

Milyaev, A., Kofler, J., Klaiber, I., Czemmel, S., Pfannstiel, J., Flachowsky, H., Stefanelli, D., Hanke, M.-V., and Wünsche, J.-N. (2021). Toward systematic understanding of flower bud induction in apple: A multi-omics approach. Front. Plant Sci. *12*, 1–18. https://doi.org/10.3389/fpls.2021.604810.

Milyaev, A., Kofler, J., Moya, Y.A.T., Lempe, J., Stefanelli, D., Hanke, M.-V., Flachowsky, H., Von Wirén, N., and Wünsche, J.-N. (2022a). Profiling of phytohormones in apple fruit and buds regarding their role as potential regulators of flower bud formation. Tree Physiol. *42*, 1–17. https://doi.org/10.1093/treephys/tpac083.

Milyaev, A., Tandron-Moya, Y.A., Von Wirén, N., Neuwald, D., Flachowsky, H., and Wünsche, J.N. (2022b). What else don't we know about biennial bearing? Phytohormone profile of seeds and seed number per fruit differ between a biennial and a non-biennial apple cultivar. Acta Hortic. *1342*, 7–14. https://doi.org/10.17660/ActaHortic.2022.1342.2.

Monselise, S.P., and Goldschmidt, E.E. (1982). Alternate bearing in fruit trees. Hortic. Rev. *4*, 128–173. https://doi.org/10.1002/9781118060773.ch5.

Mosa, W.F.A., Ali, H.M., and Abdelsalam, N.R. (2021). The utilization of tryptophan and glycine amino acids as safe alternatives to chemical fertilizers in apple orchards. Environm. Sci. Pollut. Res. *28*, 1983–1991. https://doi.org/10.1007/s11356-020-10658-7.

Murch, S.J., and Erland, L.A.E. (2021). A systematic review of melatonin in plants: An example of evolution of literature. Front. Plant Sci. *12*, 683047. https://doi.org/10.3389/fpls.2021.683047.

Pochamreddy, M., Haim, D., Halon, E., Keinan, E., Rai, A.C., Kamara, I., Sadka, A., and Irihimovitch, V. (2024). Alternate bearing in "Hass" avocado – Fruit load-induced changes in bud auxin homeostasis are associated with flowering repression. J. Experim. Bot. *75*(18), 5717–5733. https://doi.org/10.1093/jxb/erae305.

Shi, H., Wei, Y., Wang, Q., Reiter, R.J., and He, C. (2016). Melatonin mediates the stabilization of DELLA proteins to repress the floral transition in *Arabidopsis*. J. Pineal Res. *60*, 373–379. https://doi.org/10.1111/jpi.12320.

Šimura, J., Antoniadi, I., Široká, J., Tarkowská, D., Strnad, M., Ljung, K., and Novák, O. (2018). Plant hormonomics: Multiple phytohormone profiling by targeted metabolomics. Plant Physiol. *177*, 476–489. https://doi.org/10.1104/pp.18.00293.

Takagi, H., Hempton, A.K., and Imaizumi, T. (2023). Photoperiodic flowering in *Arabidopsis*: Multilayered regulatory mechanisms of *CONSTANS* and the florigen *FLOWERING LOCUS T*. Plant Commun. *4*, 1–19. https://doi.org/10.1016/j.xplc.2023.100552.

Teixeira, W.F., Fagan, E.B., Soares, L.H., Soares, J.N., Reichardt, K., and Neto, D.D. (2018). Seed and foliar application of amino acids improve variables of nitrogen metabolism and productivity in soybean crop. Front. Plant Sci. *9*, 1–12. https://doi.org/10.3389/fpls.2018.00396.

Tränkner, C., Lehmann, S., Hoenicka, H., Hanke, M.-V., Fladung, M., Lenhardt, D., Dunemann, F., Gau, A., Schlangen, K., Malnoy, M., and Flachowsky, H. (2010). Over-expression of an *FT*-homologous gene of apple induces early flowering in annual and perennial plants. Planta *232*, 1309–1324. https://doi.org/10.1007/s00425-010-1254-2.

Tromp, J. (2005). Flower-bud formation. In Fundamentals of Temperate Zone Tree Fruit Production. (Leiden, The Netherlands: Backhuys Publ.), p. 204–215.

Wójcik, P., Skorupińska, A., and Gubbuk, H. (2016). Impacts of pre- and postbloom sprays of tryptophan on calcium distribution within 'Red Jonaprince' apple trees and on fruit quality. Hortic. Sci. *51*, 1511–1516. https://doi.org/10.21273/HORTSCI11216-16.

Wójcik, P., Filipczak, J., and Wójcik, M. (2019). Effects of prebloom sprays of tryptophan and zinc on calcium nutrition, yielding and fruit quality of 'Elstar' apple trees. Sci. Hortic. *246*, 212–216. https://doi.org/10.1016/j.scienta.2018.10.071.

Zuo, X., Wang, S., Liu, X., Tang, T., Li, Y., Tong, L., Shah, K., Ma, J., An, N., Zhao, C., Xing, L., and Zhang, D. (2024). *FLOWERING LOCUS T1* and *TERMINAL FLOWER1* regulatory networks mediate flowering initiation in apple. Plant Physiol. *195*, 580–597. https://doi.org/10.1093/plphys/kiae086.

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