



# Impact of Polysorbate 20 and 80 Emulsifiers on Apple Fruitlet Thinning and their Biochemical Profile

N. Hillmayr<sup>1,2</sup> · A. Cebulj<sup>1,3</sup> · J. Jakopic<sup>2</sup> · R. Veberic<sup>2</sup> · M. Stopar<sup>1</sup>

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## Abstract

Chemical thinning is an important practise in apple production, yet current thinning agents face limitations in environmental safety and efficacy under variable conditions. This study investigates the thinning potential and physiological effects of two food-grade emulsifiers, polysorbate 20 (P20) and polysorbate 80 (P80), compared to a standard ethephon-based as well as a metamitron-based thinner on ‘Gala’ apple trees. Fruitlet drop, sugar content and phenolic profiles were monitored in relation to fruitlet position and developmental stage. Both P20 and P80 induced significant fruitlet abscission, with P20 showing a comparable thinning effect to commercial thinners. The P20 and P80 treatments altered the natural cluster hierarchy, and fruitlets with reduced growth (reduced diameter and weight) showed increased abscission, indicating lower sink strength. Sucrose content was significantly reduced in P20 treated king fruitlets, which correlated with increased fruit drop. Fructose and sorbitol content varied over time, but not between treatments, while glucose levels remained stable. Phenolic profiling revealed a significant increase in dihydrochalcones and anthocyanins after treatment with P20, suggesting a stress-induced response. These results support the carbohydrate theory of fruitlet abscission and emphasise the potential of polysorbates as alternative late-acting thinning agents with additional insights into the physiological mechanisms behind fruit drop. Further research is needed to clarify the exact mode of action of polysorbates in apple fruitlet thinning.

**Keywords** Emulsifiers · Polysorbate 20/80 · Apple fruitlet thinning · Shedding dynamic · Hierarchy · Sugars and phenolic content

## Abbreviations

P20	Polysorbate 20	NAA	$\alpha$ -naphthylacetic acid
P80	Polysorbate 80	NAD	Naphthylacetamide
ET	Ethephon based thinning agent (Ethrel)	BA	6-benzyladenine
MET	Metamitron based thinning agent (Brevis)	TMN	6-tergitol-based thinning agent
UTC	Untreated control	IAA	Indole-3-acetic acid
		DAFB	Days before full bloom
		HPLC	High-Performance Liquid Chromatography
		KF	King fruitlet
		LF	Lateral fruitlet
		HCA	Hydroxycinnamic acids
		DHC	Dihydrochalcones
		FLO	Flavonols
		FLA	Flavanols
		ANT	Anthocyanins

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✉ N. Hillmayr  
nika.hillmayr@kis.si

<sup>1</sup> Department of Fruit Growing, Viticulture and Oenology, Agricultural Institute of Slovenia, Hacquetova Ulica 17, Ljubljana SI-1000, Slovenia

<sup>2</sup> Biotechnical Faculty, Department of Agronomy, Chair for Fruit Growing, Viticulture and Vegetable Growing, University of Ljubljana, Jamnikarjeva 101, Ljubljana SI-1000, Slovenia

<sup>3</sup> Chamber of Agriculture and Forestry of Slovenia, Gospodinjska Ulica 6, Ljubljana SI-1000, Slovenia

## Introduction

Apple trees are known for their natural regulation of fruit load, with a portion of the fruit being shed. This shedding process is divided into three main periods. The first occurs 1–2 weeks after flowering and is attributed to incomplete pollination. The second, commonly referred to as the June drop, takes place 4–6 weeks after flowering and involves the most severe shedding. The third period is undesirable for fruit growers, as it involves the shedding of almost mature fruits (Luckwill 1953; Kolarič 2010). While this natural process is critical for maintaining fruit quality and tree health, the amount of fruit loss is often insufficient, leading to the need for chemical thinning (Valentini and Arroyo 2002). Alternate bearing in which trees produce a heavy crop one year and a light crop the next is also a common issue in apple orchards, further motivating the need for effective chemical thinning practices (Tromp 2000).

Currently registered and commonly used chemical apple thinners have certain limitations. Thinning efficacy depends on the application rate, timing relative to the fruit developmental stage, and prevailing weather conditions, which together influence the tree's carbohydrate balance and fruitlet sensitivity to abscission (Lakso et al. 2007; Gonzalez et al. 2023). Temperature is one of the most critical factors and can limit the efficiency of  $\alpha$ -naphthylacetic acid (NAA), benzyladenine (BA) or ethephon-based agents, with cooler conditions generally reducing and warmer conditions enhancing fruit abscission (Robinson and Lakso 2004; Parra-Quezada et al. 2006; Yuan 2007; Kviklys and Robinson 2010). In addition to temperature, solar radiation influences the thinning efficacy of NAA and BA indirectly through its effect on tree carbohydrate balance (Robinson and Lakso 2004; Lakso et al. 2006). Metamitron efficacy is also highly dependent on environmental conditions, as reduced solar radiation and elevated night temperatures increase its thinning efficiency (Tadmor et al. 2021; Gonzalez et al. 2023). Occasionally, toxicological concerns associated with thinning agents may arise. As awareness of the toxic effects of certain chemicals on the environment grew, one of the first thinners, carbaryl, was banned in Europe due to its incompatibility with the environment (Gunasekara et al. 2008). Long-term toxicological safety can be expressed using the Acceptable Daily Intake (ADI), defined as the amount of a chemical substance that can be ingested daily over a lifetime without appreciable risk to health, expressed on a body-weight basis ( $\text{mg kg}^{-1} \cdot \text{body weight day}^{-1}$ ) (Chilakapati and Mehendale 2014). Thinning agents generally show low long-term toxicological concern; for example, NAA has an ADI between 0 and  $0.15 \text{ mg kg}^{-1} \text{ bw day}^{-1}$  and ethephon has an ADI of  $0\text{--}0.05 \text{ mg kg}^{-1} \text{ bw day}^{-1}$  (Wang and Hao 2023). However, food additives such as certain

emulsifiers (Polysorbates 20 and 80) have substantially higher ADI values, ranging from 0 to  $25 \text{ mg kg}^{-1} \text{ bw day}^{-1}$ , meaning they can be consumed in larger quantities compared to thinning agents (WHO 2021a; WHO 2021b).

As there is no perfect apple fruitlet thinning agent on the market, research is focused on finding more reliable and environmentally friendly thinners. Fallahi et al. (2006) have demonstrated effective thinning of peaches, nectarines and plums with a tergitol-based thinning agent (TMN-6), which belongs to the group of surfactants. Similarly, Klein and Cohen (2000) demonstrated the multifunctional activity of the organosilicone emulsifiers Silwet-408 and Boost in nectarines and peaches. Silwet was later also tested in thinning of apples, on the 'Jonagold', where positive results were also obtained (Bound and Klein 2010). Lañar et al. (2022) showed that thinning plums with SilwetStar was successful as number of fruits per tree was reduced by 69%. This led to the idea of testing other emulsifiers and their thinning effect. Some emulsifiers are defined by Codex Alimentarius as food additive, which forms or maintains a uniform emulsion of two or more phases in food (Codex Alimentarius 2015). Polysorbate 20 (E432) and polysorbate 80 (E433) are a class of surface active substances so called emulsifiers. Polysorbates act as emulsifiers due to their amphipathic structure, which contains both hydrophilic polyoxyethylene chains and hydrophobic fatty acid esters, allowing them to adsorb at the oil–water interface. By reducing interfacial tension and preventing droplet coalescence, polysorbates stabilize oil-in-water emulsions, and their non-ionic nature makes this effect independent of pH and ionic strength (Kerwin 2008). Polysorbates are a food additive, for example polysorbate 80 is the most common of the sorbitan esters used for ice cream applications (Euston and Goff 2019). In our earlier work we focussed on thinning effect of polysorbates. The double application of polysorbates at a fruit diameter of 15–22 mm showed positive results in reducing the crop load of apple trees (Stopar and Hladnik 2020, 2021; Hillmayr and Stopar 2024).

Dominance within the fruitlet cluster plays a key role in both natural shedding and chemically induced thinning. The central flower (the so-called "king" flower) in the cluster is the first to bloom, thus assuming the dominant role and forming a clear hierarchy within the cluster (Bangerth 2000; Botton et al. 2011; Jakopic et al. 2015). This phenomenon is also known as "primigenic dominance" (Bangerth 1989). It means that the fruit that sets first has a stronger sink force and is more likely to reach maturity, while the lateral fruits (LF; L1 - L5), especially those that are distant from the king fruit, are more likely to abscise (Bangerth 2000; Botton et al. 2010). There are two main theories to explain the abscission of the young fruit. The first, the carbohydrate theory, states that the fruitlets did not receive enough assimilates

because either too few assimilates were produced or too few were distributed to the fruitlet cluster (Lakso and Gofinet 2013; Jakopic et al. 2016). An alternative view is that fruit shedding is due to hormonal mechanisms (Bangerth 2000). Researchers support both theories and many studies have been done to confirm them, but the mechanism behind abscission of each commercial thinning agent is still unknown.

The aim of our study is to understand how polysorbates, when thinned with them, affect the abscission process of apple fruitlet. Furthermore, we want to see whether the dominance in fruitlet clusters changes and how polysorbates influence the frequency or intensity of shedding. Primary and secondary metabolites as indicators of changes in plants were also analysed. Certain phenolic compounds increase under different stress conditions (Dixon and Paiva 1995). The possible use of polysorbates as thinning agents has been researched (Stopar and Hladnik 2020, 2021; Hillmayr and Stopar 2024). However, their mode of action has not yet been detriment. Detailed knowledge on mode of action of polysorbates as thinning agents could help to use them in apple orchard as plant bioregulators.

## Materials and Methods

### Plant material and Experimental Design

This study was conducted in 2023 on 11-year-old trees and in 2024 on 7-year-old trees of the ‘Brookfield Gala’ apple clone grafted onto M.9 rootstock, growing in the experimental orchard at Brdo pri Lukovici (46° 10’ 04.8” N, 14° 40’ 55.2” E). The trees were planted at a distance of 3.2 × 1 m and trained as slender spindles with a canopy height of 1.8–2 m. The rows were orientated in a N/NE-S/SW direction and the inter-row space was covered by permanent grass cover. The orchard was equipped with hail net, trees were supported by bamboo sticks and there was no irrigation system. Trees with uniform vigour and flowering intensity were selected (Table 2). The experiment was set up in a randomised complete block design with eight blocks,

four treatments (untreated control, ethephon, polysorbate 20 and polysorbate 80) in 2023 and five treatments (untreated control, ethephon, metamitron, polysorbate 20 and polysorbate 80) in 2024. Each treatment was applied to two trees per block, resulting in a total of 64 (in 2023) and 80 (in 2024) trees, respectively. Border trees were used between treatments. In each pair, one tree was used to collect fruitlet samples and the other to measure the frequency of fruitlet drop. All spraying treatments were applied to plots with individual trees using a backpack sprayer up to the point of drip; 300 mL of water was used per tree, i.e. 1000 L ha<sup>-1</sup> of water consumption.

### Treatments

Treatments, concentrations and application timings are presented in Table 1. The study was conducted in 2023 with four treatments: untreated control (UTC), chemical thinning with ethephon (ET, Ethrel, Bayer, Leverkusen, Germany), and two alternative thinners, polysorbate emulsifiers – polysorbate 20 (P20, polyoxyethylene (20) sorbitan monolaurate, Tween 20, Merck KGaA, Darmstadt, Germany) and polysorbate 80 (P80, polyoxyethylene (20) sorbitan monooleate, Tween 80, Merck KGaA, Darmstadt, Germany). In 2024, an additional treatment was included: chemical thinning with metamitron (MET, Brevis, Adama, Leusden, Netherlands). Ethrel contained 480 g L<sup>-1</sup> of ethephon, Brevis contained 150 g kg<sup>-1</sup> of metamitron in the product, and both Tween 20 and Tween 80 were 100% active agent. Control trees were left untreated. Ethephon was applied once when the fruitlet diameter reached 12 mm, which was 27 days after full bloom (DAFB) in 2023 and at diameter 11 mm on 27 DAFB in 2024. Metamitron was applied once when the fruitlet diameter reached 12 mm, which was 27 DAFB in 2024. Polysorbates 20 and 80 were each applied twice: the first application was at a fruitlet diameter of 12 mm (27 DAFB) in 2023 and 11 mm (27 DAFB) in 2024, followed by a second application when the fruitlet diameter reached 19 mm (31 DAFB) in 2023 and 20 mm (38 DAFB) in 2024. Meteorological data for the experimental period are presented in Supplementary Figures S1 and S2. In both years, thinning

**Table 1** Overview of thinning treatments, concentrations, and application timing based on KF diameter and DAFB during the 2023 and 2024 growing seasons

Treatment	Concentration of active agent	Concentration of used product	2023	2024
			Application time	Application time
Untreated control(UTC)	–	–	–	–
Ethephon (ET)	300 mg L <sup>-1</sup>	630 µL L <sup>-1</sup> of Ethrel	KF 12 mm on 27 DAFB	KF 11 mm on 27 DAFB
Metamitron (MET)	160 mg L <sup>-1</sup>	1,1 g L <sup>-1</sup> of Brevis	–	KF 11 mm on 27 DAFB
Polysorbate 20 (P20)	5 mL L <sup>-1</sup>	5 mL L <sup>-1</sup> of Tween 20	1. KF 12 mm on 27 DAFB 2. KF 19 mm on 31 DAFB	1. KF 11 mm 27 DAFB 2. KF 20 mm 38 DAFB
Polysorbate 80 (P80)	5 mL L <sup>-1</sup>	5 mL L <sup>-1</sup> of Tween 80	1. KF 12 mm on 27 DAFB 2. KF 19 mm on 31 DAFB	1. KF 11 mm on 27 DAFB 2. KF 20 mm on 38 DAFB

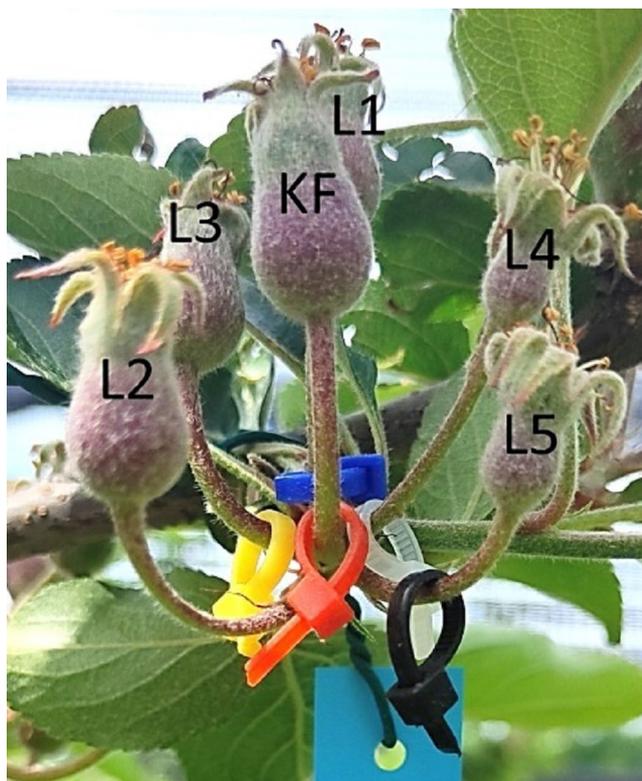
KF, king fruit; DAFB, days after full bloom.

treatments were applied under dry conditions, with leaves remaining dry for at least 10 h prior to application.

## Investigation

As mentioned above, the same treatments were carried out on two paired trees, one for monitoring fruit drop and the second for all remaining assessments. For fruit drop monitoring, two fruit clusters were selected on opposite sides of the canopy on each tree. Fruitlet clusters were located in the centre of the canopy (approximately 100–150 cm above the ground) and at the edge of the tree canopy. The hierarchy in each cluster was marked with coloured rings, from the king fruitlet (KF) in the central position to the lateral fruitlets (LF), where L1 was the fruit closest to the king fruitlet and L5 was the fruitlet furthest from the KF (Fig. 1). Fruit drop was monitored in both years and the diameter of each labelled fruitlet was measured 17 times between 12 DAFB and harvest (129 DAFB) in 2023 and 25 times between 16 DAFB and harvest (139 DAFB) in 2024. At harvest number of fruit per tree (No. fruit tree<sup>-1</sup>) was counted in both years.

Fruitlets for chemical analyses were collected in 2023 from each tree at four different times: 27 May 2023 (29 DAFB, 3 days after treatment (DAT)); 2 June 2023 (35 DAFB, 9 DAT); 7 June 2023 (40 DAFB, 14 DAT); and 12 June 2023 (45 DAFB, 19 DAT). Two random fruitlet clusters



**Fig. 1** Monitored positions in the fruit cluster, king fruitlet (KF) and lateral fruitlets (L1–L5) from the top to the base

were taken from each tree for HPLC (High-Performance Liquid Chromatography) analysis and one random fruit cluster for fresh and dry weight analysis. The criteria for sampled fruitlets were that they did not show any signs of abscission (yellowing or dehydration). Secondly, to surely distinguish between KF and LF we only sampled L2–L5 as representative of LF group and not L1, which can sometimes act like KF. In the laboratory, the samples from two blocks were combined so that four representative blocks were available for laboratory analysis. The content of individual sugars and phenolic compounds was determined.

## Analysis of Individual Sugars

The sugar content was determined according to Jakopic et al. (2016) with slight modifications. To analyse the sugars, 5 g of fruitlets were homogenised in 25 mL of double distilled water using a T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). The sugars were extracted at room temperature for 30 min on a shaker Unimax 1010 (Heidolph, Schwabach, Germany) and then the mixture was centrifuged for 10 min at 10,000 rpm (Eppendorf Centrifuge 5810 R, Hamburg, Germany). The supernatant was filtered through 0.2 µm cellulose mixed ester filter (Macherey-Nagel, Düren, Germany) and transferred to a vial for analysis.

HPLC analyses were performed on a Vanquish HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with refractive index (RI) detector for sugar identification. A 20 µL sample was injected onto a Phenomenex Rezex RCM monosaccharide column (150 × 7.8 mm; Phenomenex, Torrance, USA) operating at 85 °C with a flow rate of 0.8 mL/min. Double distilled water served as the mobile phase. Sugars were identified and quantified by comparing retention times and concentrations with external standards. Results were expressed in g per kg dry weight (DW).

## Analysis of the Phenolic Compounds

The individual phenols were also analysed with slight modifications of the protocol by Jakopic et al. (2016). Prior to analysis, fruitlets were chopped into small pieces and a sample of 5 g was extracted with 10 mL methanol containing 3% (v/v) formic acid in a cooled ultrasonic bath for one hour. The treated samples were centrifuged at 10,000 rpm for 10 min. The supernatant was filtered with a 0.2 µm polyamide filter (Macherey-Nagel, Düren, Germany), the filtrate was transferred to a vial and injected into the HPLC system.

Chromatographic analysis was performed using a Thermo Scientific Vanquish HPLC system (Thermo Scientific, Waltham, MA, USA) coupled to a photodiode array detector set at 280 nm (detection of flavanols, hydroxycinnamic acids and dihydrochalcones) and 350 nm

(detection of flavonols). A Gemini Phenomenex C18 column (150 × 4.6 mm, 3 $\mu$ ; Phenomenex, Torrance, USA) at a temperature of 25 °C was used. The flow rate was 0.6 mL/min. The eluents were 0.1% (v/v) water-based formic acid with 3% acetonitrile (A) and 3% double distilled water with 0.1% formic acid in acetonitrile (v/v/v) (B). Samples were eluted with a gradient: from 5% B to 20% B over the first 15 min, 15–20 min linear increase to 30% B, 20–25 min hold at 30% B, 25–30 min increase to 90% B, 30–35 min increase to 100% B, 35–40 min hold at 100% B, 40–45 min, re-equilibration at 5% B.

The compounds were characterised by comparing the UV–vis spectra and retention times of standards and confirmed by mass spectrometric analysis (HPLC-MS). The later was performed on a Thermo Scientific LTQ XL linear ion trap with an electrospray interface (ESI) source in negative mode (Thermo Scientific, Waltham, MA, USA). Full-scan MS spectra were acquired over  $m/z$  115–1000 and data-dependent MS/MS was applied for structural confirmation. Data acquisition and processing were performed with Xcalibur software (Thermo Scientific). Quantification was determined using the concentrations of a suitable external standard and the individual phenolic compounds were expressed in g/kg DW.

Several compounds were detected in developing fruitlets and they were arranged in five phenolic groups as followed: flavanols (procyanidins B1 and B2, catechin, procyanidin dimer and trimer epicatechin), dihydrochalcones (phloretin 2-*O*-xylosylglucoside, phloretin 2'-*O*-glucoside, 3-hydroxyphloretin-2-*O*-xyloside, phloretin-diglucoside), hydroxycinnamic acids (*p*-coumaric acid derivatives, caffeic acid glucoside, caffeic acid derivatives, *p*-coumaroyl hexoside, 4-caffeoylquinic acid, quinic acid derivative, 5-*O*-caffeoylquinic acid, ferulic acid derivative, 3-*O*-caffeoylquinic acid, 4-*O*-*p*-coumaroylquinic acid (trans and isomere cis), 5-*O*-*p*-coumaroylquinic acid and chlorogenic acid), flavonols (quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-rhamnoside) and anthocyanins (cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-7-*O*-arabinoside and cyanidin-3-*O*-xyloside).

## Chemicals

For the quantification of sugars the following standards were used: fructose, glucose, sucrose and sorbitol from Fluka (Buchs, Switzerland). The following standards used for calibration of phenolic compounds were: procyanidin B2, cryptochlorogenic acid, neochlorogenic acid, quercetin-rutinoside, quercetin-galactoside, chlorogenic acid, caffeic acid, quercetin-arabinofuranoside and cyanidin-glucoside

from Sigma Aldrich (Germany), ferulic acid, *p*-coumaric acid, phloridzin, catechin and epicatechin from Fluka Chemie GmbH (Buchs, Switzerland). The chemicals for the mobile phases were HPLC-MS grade acetonitrile from Merck (Darmstadt, Germany) and formic acid from Honeywell (Seelze, Switzerland). Water for the mobile phase was twice distilled and purified with a Mili-Q Millipore Direct 8 system (Merck Millipore, Billerica, MA, USA).

## Statistical Analysis

Initial flower cluster density (No. FC tree<sup>-1</sup>) data and crop load data (No. fruit tree<sup>-1</sup>) were analysed using one-way ANOVA followed by Duncan's multiple range test. Fruitlet drop data were analysed using survival analysis methods. For each fruitlet a position in fruit cluster was determined as well as the lifespan, which was either the time difference between full bloom and fruitlet drop or time difference between full bloom and harvest (in this case the fruitlet survived until the end of observation period). The survival curves were constructed using the Kaplan-Meier method with strata being fruitlet position and treatment. Survival curves were compared statistically using the Cox proportional hazard model. The fruitlet diameter data and sugar content data were analysed using a linear effects model, followed by Tukey-adjusted comparisons of estimated marginal means. Fruitlet weight data were analysed using factorial ANOVA, while treatment differences were evaluated using Tukey-adjusted pairwise comparisons of estimated marginal means. The phenolic content data were analysed using repeated measures ANOVA, followed by Tukey-adjusted comparisons of estimated marginal means. All statistical analysis was done in R Studio (R Project for Statistical Computing, Auckland, New Zealand). Results were expressed as mean values. Statistical significance was determined at the  $\alpha$  significance level, where  $\alpha=0.05$ .

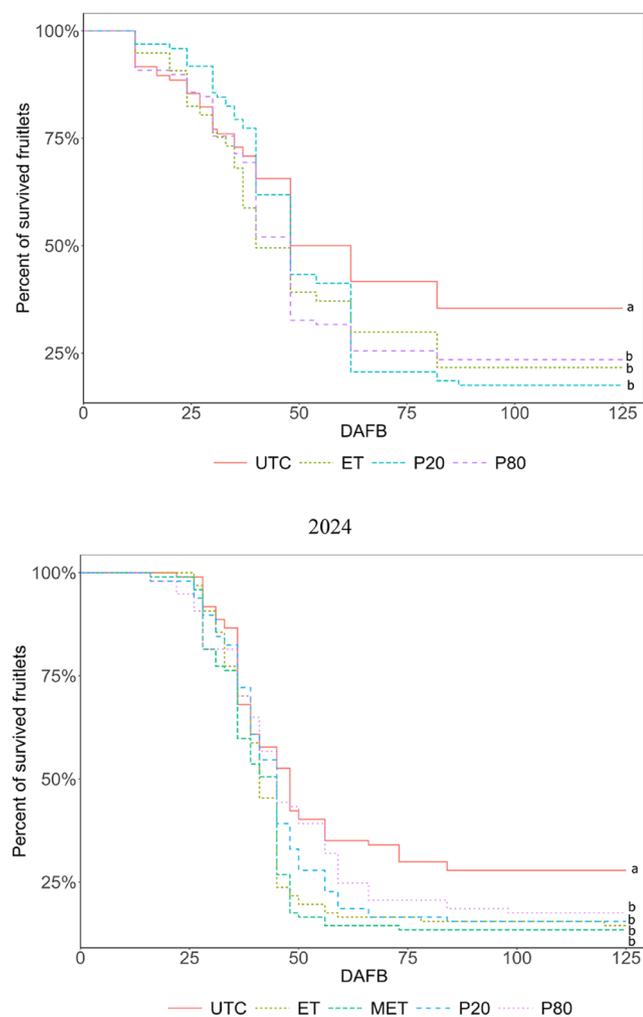
## Results

### Thinning Effect

Thinning effect on fruitlets independent of their position in the cluster was observed with all thinning agents used (ET, MET, P20, P80) in years 2023 and 2024. At the start of the experiment, the number of flower clusters per tree did not differ significantly among treatments in either year, indicating uniform initial flowering (Table 2). In contrast, the number of fruits per tree was significantly reduced by all thinning treatments compared with the untreated control in both 2023 and 2024, with the strongest thinning effect observed for P20 in both years (Table 2). In both years,

**Table 2** Comparison of the number of flower clusters (FC) per tree and the number of fruits per tree among treatments in 2023 and 2024. Values are presented as means  $\pm$  standard errors. Different letters within a column indicate significant differences among treatments at  $p < 0.05$ 

	2023		2024	
	No. FC tree <sup>-1</sup>	No. fruit tree <sup>-1</sup>	No. FC tree <sup>-1</sup>	No. fruit tree <sup>-1</sup>
UTC	130.9 $\pm$ 7.1a	153.1 $\pm$ 15a	150 $\pm$ 5.54a	165.75 $\pm$ 7.89a
MET			146.75 $\pm$ 6.16a	129.88 $\pm$ 14.86b
ET	131 $\pm$ 4.4a	96.6 $\pm$ 12b	150.125 $\pm$ 3.52a	120.00 $\pm$ 6.80b
P20	130.1 $\pm$ 4a	76.1 $\pm$ 7.7b	150.125 $\pm$ 7.52a	71.50 $\pm$ 10.88c
P80	126.6 $\pm$ 5.2a	107.9 $\pm$ 14b	148.125 $\pm$ 5.97a	115.88 $\pm$ 11.43b

**Fig. 2** Comparison of fruit drop between treatments independent of position in 2023 (first figure) and 2024 (second figure). Letters indicates differences between treatments (ET, MET, P20 and P80) and UTC at  $p < 0.05$ 

shedding was more severe when thinning agent was used compared to the untreated control (UTC), where 35% of the monitored fruitlets remained in 2023 and 28% in 2024 (Fig. 2). Intense fruit drop was observed between the 10th and 45th DAFB in 2023, but in 2024 it occurred later, from 25th to 50th DAFB. In 2023 the most effective thinning agent was P20 (18% of the fruitlets survived), followed by

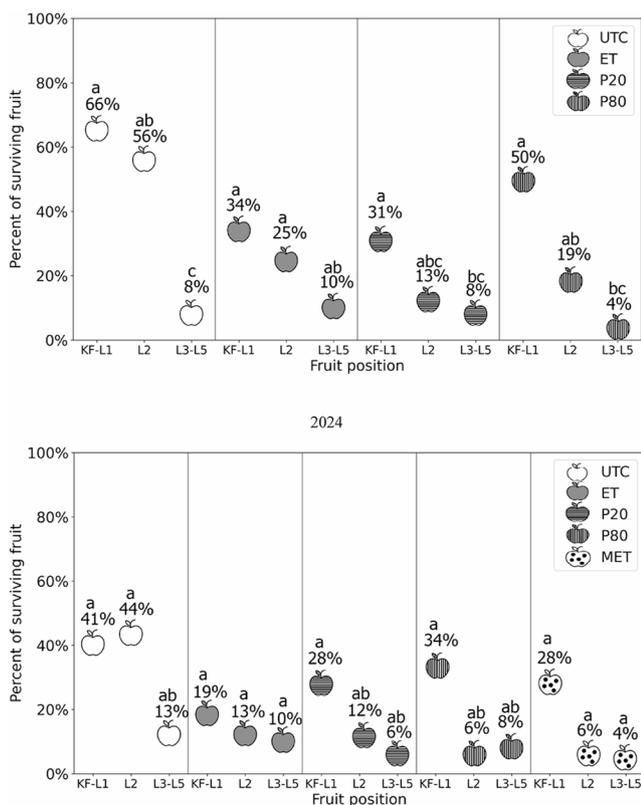
ET (22% of the fruitlets survived) and finally P80 (24% of the fruitlets survived). However, in 2024 metamitron was most effective (13% of the fruitlets survived), followed by ET (14% of the fruitlets survived), then P20 (16% of the fruitlets survived) and finally P80 (17% of the fruitlets survived). Significantly fewer fruitlets survived when a thinning agent was used than with UTC in both years.

Figure 3 also shows the thinning effect in both years with the final percentage of survived fruit in the KF-L1, L2 and L3-L5 groups right before harvest. In both years, the percentage of surviving fruit was lower in the lower hierarchy groups, such as L3-L5. Notably, KF-L1 had the highest survival rate at UTC in 2023. In 2024, fruit drop at UTC was generally more uniform for all three groups (KF-L1, L2 and L3-L5). On the other hand, a similar fruit drop rate was observed for ET and P20 in both years. In addition, P80 had the highest survival rate in both years; in 2023, KF had the highest survival rate, but in 2024 it was L2.

### Frequency of Thinning and Growth of Fruitlets within the Cluster

The survival curves for all four treatments in 2023 are shown in Fig. 4. In the untreated control (Fig. 4a), we found no difference between KF, L1 and L2, which formed a group with less fruit drop. But, there was a statistical difference between the groups L1, L2, KF and L4, L5, which means that L4 and L5 were more likely to abscise than L1, L2 and KF. In 2024, however, there were only two statistical differences: the UTC survival curve of L3 differed significantly from that of L1 and L2 (Fig. 5a).

In the ET treatment (Fig. 4b), there were statistically significant differences between L5 and the other fruitlets in the cluster in 2023. In 2024, however, the fruit drop in the cluster was more homogeneous without being statistically significant (Fig. 5b), as was also the case with MET, which was only applied in 2024 (Fig. 5e). Figure 4c shows the survival curves when P20 was used in 2023. The statistically significant difference was between KF-L5, L1-L5, L3-L5, L1-L4 and L1-L5, meaning that L4 and L5 were more likely to abscise. In 2024, L5 was also keener to drop than KF and L1, when sprayed with P20 (Fig. 5c). Finally, in treatment P80 in 2023 (Fig. 4d), L5 and partially L4 had a greater



**Fig. 3** Percent of survived fruit groups on positions KF-L1, king fruit and first lateral fruit; L2, second lateral fruit and L3-L5, lateral fruits at 3th, 4th and 5th positions on 125 DAFB (i.e. 4 days before harvest) in 2023 (first figure) and on 139 DAFB (i.e. day of harvest) in 2024 (second figure). Treatments: blank, untreated control; grey, thinned with ethephon; horizontal stripes, thinned with polysorbate 20; vertical stripes, thinned with polysorbate 80 and dotted, thinned with metatmitron. Letters indicates differences between fruit positions within the same treatment at  $p < 0.05$

chance of abscising than KF, L1 and L2, however in 2024, this only applied to L4 and not L5.

In general, two groups of fruitlets were identified in 2023 according to the position in the cluster. KF-L1 abscised less than the L3-L5 group, regardless of the thinning agent used. Similarly, in 2024, the KF-L1 group was less likely to drop.

The diameters and weights of the fruitlets also depended on their position in the hierarchy and the treatment applied. The diameters and weights of the individual fruitlet groups in 2023 are shown in Figs. 6 and 7. Treatment P80 significantly increased the diameter and growth rate of the king fruitlet (Fig. 6a), with KF being on average 10%, 14% and 21% larger than in UTC, ET and P20, respectively. KFs of remaining treatments did not differ significantly from each other.

The thinning treatments were more noticeable in the LFs, in 2023 significantly stunted growth of the first lateral L1 was observed in 29 DAFB in treatments P20 and P80 compared to UTC (Fig. 6b). This was even more evident

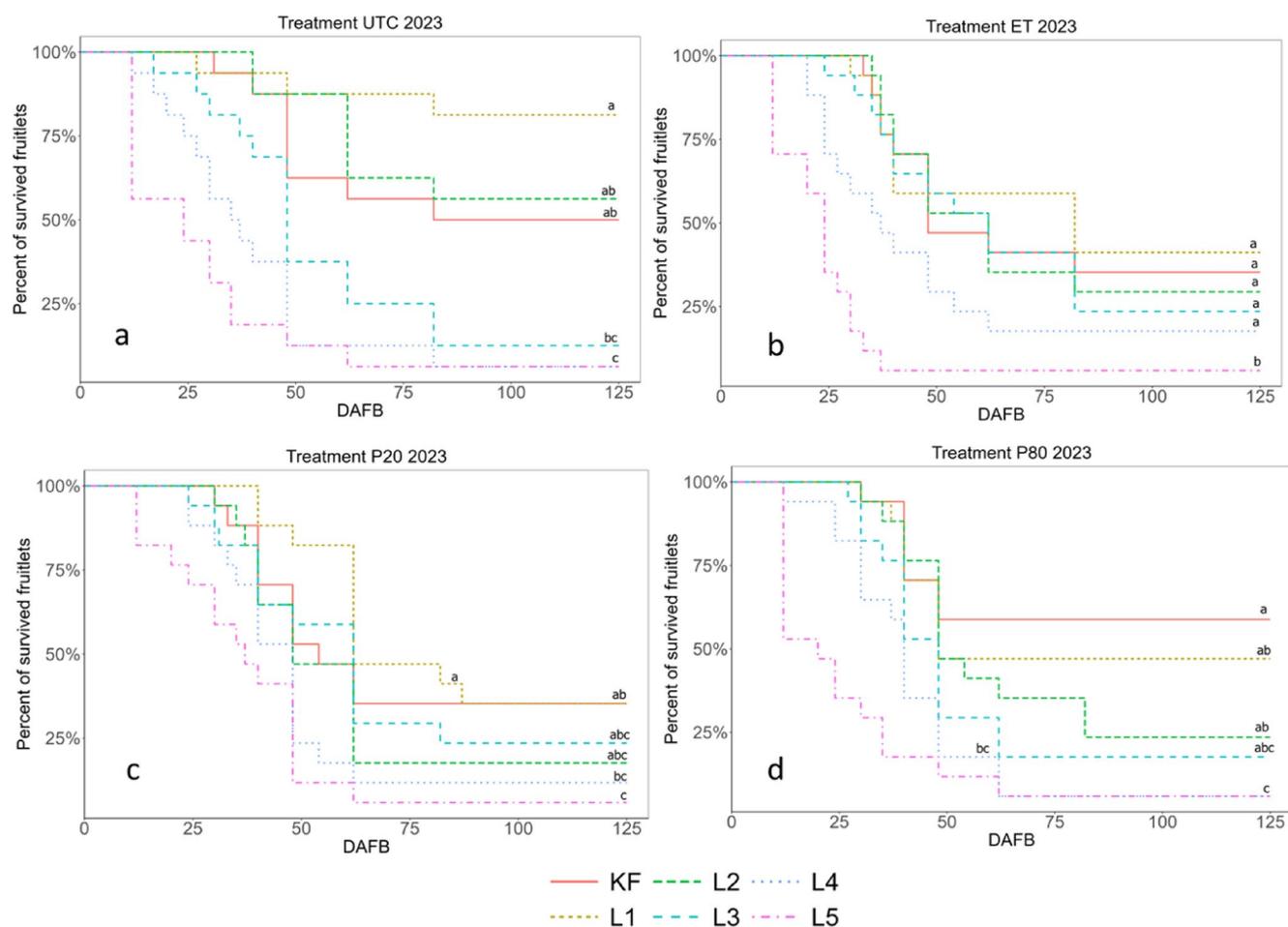
at the L2 position, where the P20 and P80 fell even further behind the untreated fruitlets (UTC). The P20-treated L2 was also significantly smaller than the ET and P80-treated L2 (Fig. 6c). The fruitlets at positions L3-L5 treated with P80 were significantly smaller in diameter compared to all other treatments, whereas P20-treated fruitlets exhibited intermediate diameters, being significantly larger than P80 but smaller than UTC- and ET-treated fruitlets (Fig. 6d). In addition, fruitlets groups L2 and L3-L5 had significantly larger diameters in 2023 when thinned with P20 compared to P80.

Statistical analysis showed that fruitlet fresh weight in 2023 was significantly affected by the applied treatment, fruitlet position, and sampling date (Fig. 7). When averaged across sampling dates, the weight of KF was significantly lower under treatment P20 compared to UTC, whereas no significant differences were observed between UTC and ET or P80 treatments (Fig. 7a). For LF pooled across positions L2-L5, fruitlets treated with P80 and P20 had significantly lower fresh weight compared to UTC, while ET-treated fruitlets had intermediate values (Fig. 7b). Across all treatments, LF were significantly lighter than KF, indicating a strong effect of fruitlet position on fresh weight.

### Sugar Content in the Surviving King and Lateral Fruitlets

The sugar content was monitored during the shedding of fruitlets in 2023. The upward or downward trend of the content of each individual sugar is similar in king and lateral position of fruitlet. The content of each sugar for all treatments in 2023 (UTC, ET, P20 and P80) is shown in Fig. 8. Glucose and fructose were most prevalent in both KF and LF. There was no difference between the treatments in terms of glucose and fructose content, neither in KF nor in LF. The glucose content in both fruitlets types (KF and LF) remained stable over the investigation periods (Fig. 8b).

A significant treatment  $\times$  time interaction was observed in the KF group (Fig. 8a1), indicating that P20 resulted in significantly lower sucrose content compared with UTC at the third and fourth investigation dates (40 and 45 DAFB). At the second investigation date (35 DAFB), a marginal reduction in sucrose content in KF was observed for P20 relative to UTC ( $p < 0.1$ ), but this difference was not statistically significant. However, no significant treatment differences were detected at the first investigation date. In group LF a marginal treatment  $\times$  time interactions were observed, indicating a tendency toward lower sucrose content in P80 at the second investigation date and in both P20 and P80 at the fourth investigation date compared with UTC ( $p < 0.1$ ). These effects should be interpreted as trends rather than statistically significant differences. The sorbitol content



**Fig. 4** Survival curves for the tested treatments in relation to the position of the fruitlets (KF – L5) within the flower cluster in 2023 (a, untreated control; b, thinned with ethephon; c, thinned with polysor-

bate 20 and d, thinned with polysorbate 80). Different letters indicate significant differences among survival curves within each fruit cluster ( $p < 0.05$ )

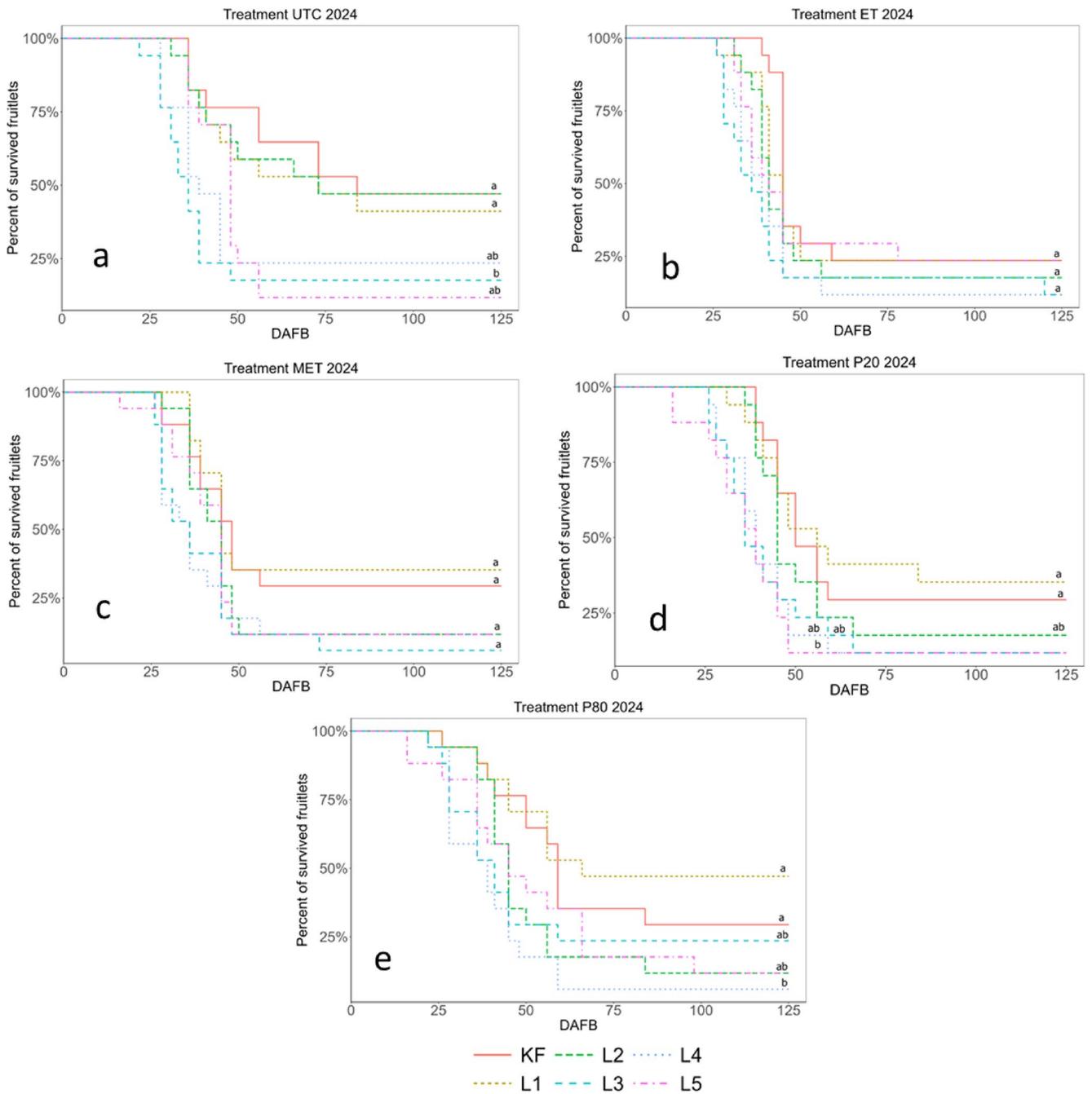
decreased during shedding in both the central and lateral positions. No difference was observed between the treatments, neither in KF nor in LF. No noticeable change was observed in the sum of sugars.

### Phenolic Content In The Surviving King And Lateral Fruitlets

In 2023 the levels of phenolic compounds were determined during the shedding period; phenols were grouped and are shown in Fig. 9 as five groups of phenolics: hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols and anthocyanins. The trend for each phenolic group is similar for king and lateral positions of fruitlets.

The content of flavanols (Fig. 9a) did not differ between the different treatments, neither in the KF nor in the LF. However, there was a marginal difference between the content trends for UTC and P20 in KF and LF. Dihydrochalcones content was significantly different in the trends between the UTC and P20 treated treatments for fruitlets

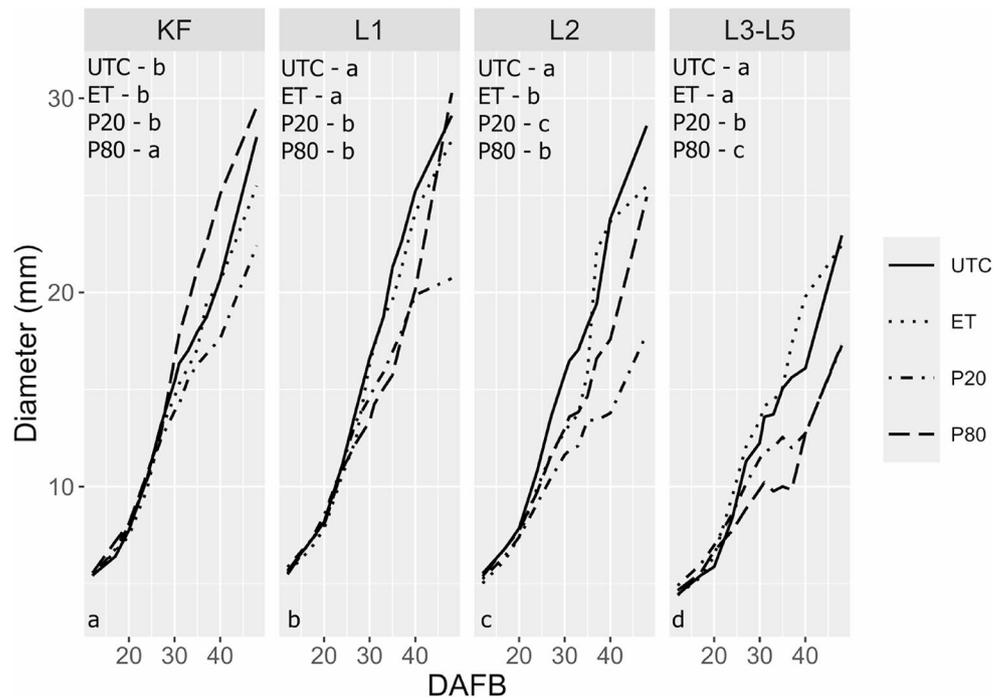
in the king and lateral positions (Fig. 9b). In both positions, the P20 treatment showed higher levels of dihydrochalcones in the fruitlet in the last three investigations. Overall, the dihydrochalcones content decreased during the observed period. Hydroxycinnamic acids and flavonols content also decreased over the four investigation time point, but no significant difference was observed between treatments, either at the KF position or at the LF position (Fig. 9c-d). In contrast, the anthocyanins content decreased up to the first investigation time point (29 DAFB), but at the next three investigation time points (35–45 DAFB), the anthocyanins content more or less increased in the fruitlets in the KF and stagnated in the fruitlets at the lateral positions. In addition, some significant differences were observed between treatments in terms of anthocyanins content development (Fig. 9e). On first sampling date ET and P20 treated treatments had significantly higher content of anthocyanins in the KF than the UTC. In KF, the anthocyanins trends at P20 and P80 were also different, in favour of P20 (Fig. 9e1). The same results were repeated for fruitlets in the lateral



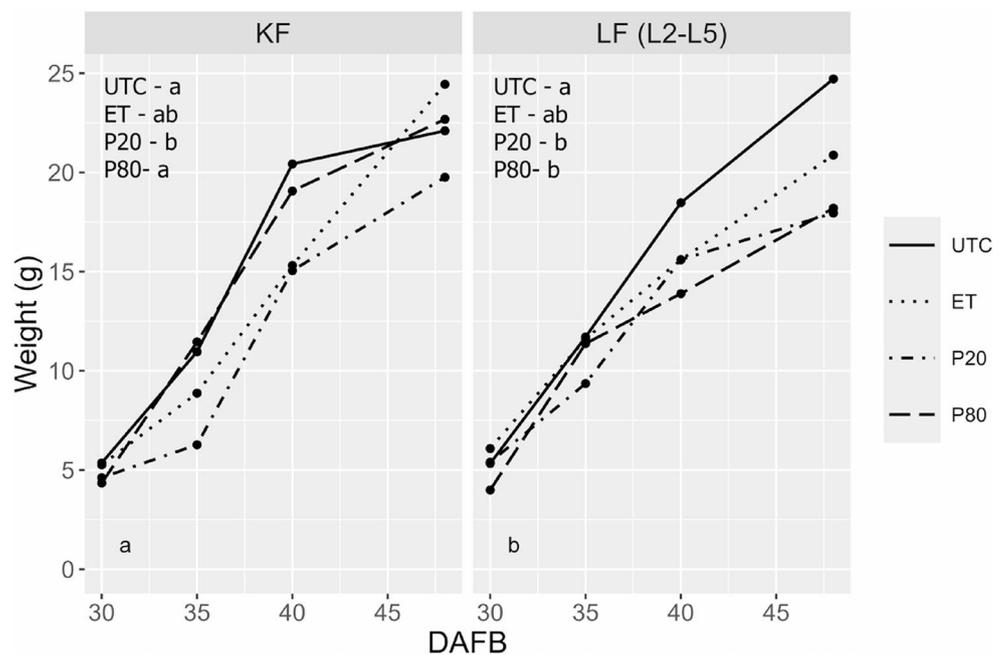
**Fig. 5** Survival curves for the tested treatments in relation to the position of the fruitlets (KF – L5) within the flower cluster in 2024 (a, untreated control; b, thinned with ethephon; c, thinned with polysor-

bate 20; d, thinned with polysorbate 80 and E, thinned with metamitron). Different letters indicate significant differences among survival curves within each fruit cluster ( $p < 0.05$ )

**Fig. 6** Diameter of apple fruitlets during shedding period. KF, king fruitlet (a); L1, first lateral fruitlet (b); L2, second lateral fruitlet (c); L3-L5, lateral fruitlets on positions 3–5 (d) in 2023. Different letters indicate significant differences among treatments within each fruitlet position (Tukey test,  $p < 0.05$ ); letters refer to treatment effects averaged across measurement dates



**Fig. 7** Fresh weight of apple fruitlets that did not abscise during shedding period. KF, king fruitlet (a) and LF (L2-L5), lateral fruitlets, position from L2 to L5 (b) in 2023. Different letters indicate significant differences among treatments within each fruitlet group (Tukey test,  $p < 0.05$ ); letters refer to treatment effects averaged across sampling dates

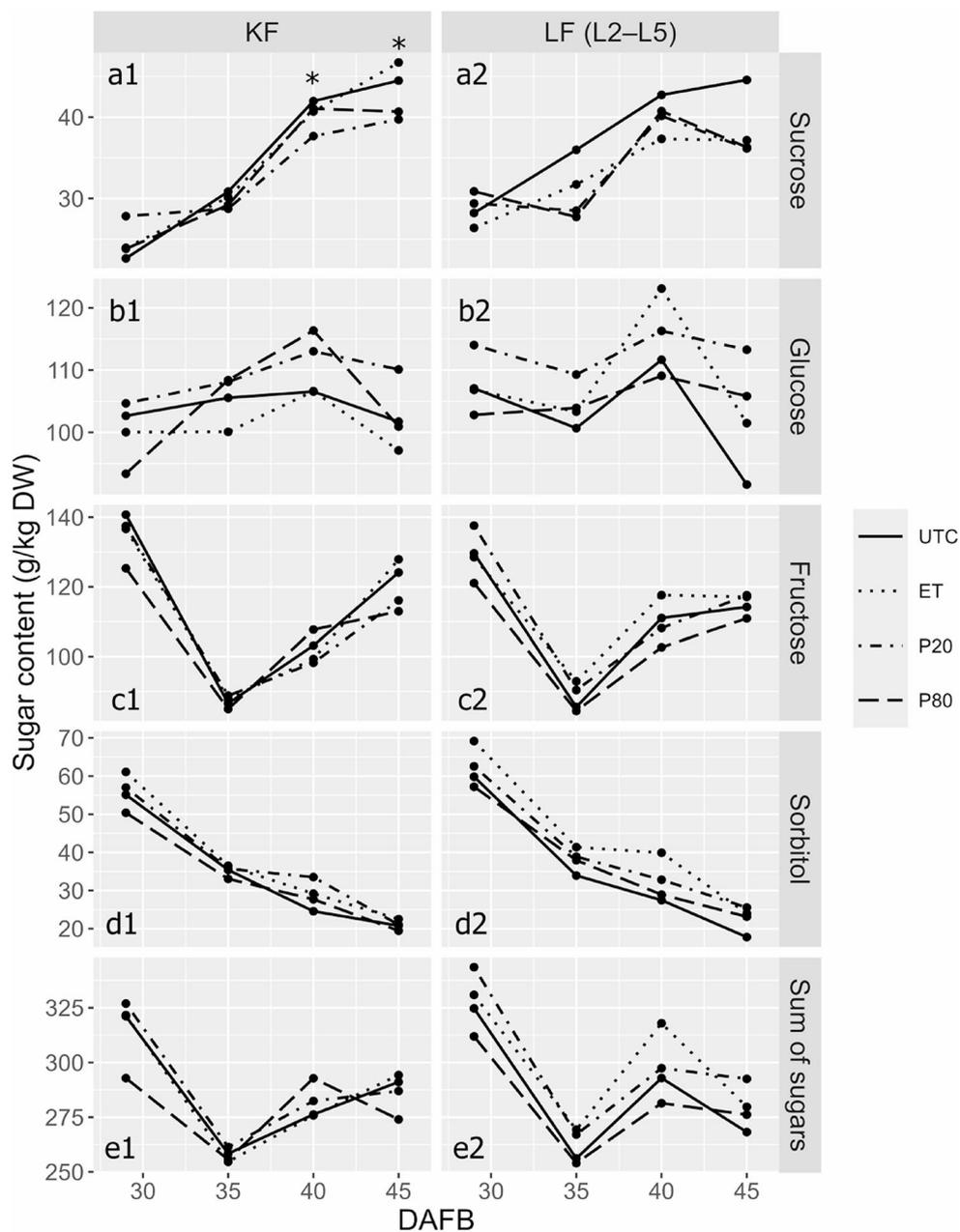


positions, where the UTC treatment was significantly lower than ET and P20, while P20 was again significantly higher than P80 (Fig. 9e2). No remarkable change was observed in the sum of phenolics, the trend was similar to the flavanols, which were the dominant phenols in both KF and LF, while the groups of dihydrochalcones, flavonols and anthocyanins were only present in smaller amounts.

## Discussion

Polysorbates 20 and 80 proved to be effective alternative thinning agents for apple fruitlet thinning in both test years. In 2023 and 2024, the thinning efficiency of P20 and P80 was comparable to the standard thinning agents ethephon (Meland and Kaiser 2011) and metamitron (Gonzalez et al. 2020). In 2024 the thinning effect of P80 was weaker than that of P20. P20 had a stronger thinning effect than ethephon in both years and metamitron in 2024 (Table 2).

**Fig. 8** The content of individual sugars: sucrose (a); glucose (b); fructose (c); sorbitol (d) and the sum of sugars (e) in the apple fruitlets during the shedding process. KF, king fruitlets; LF (L2–L5), lateral fruitlets, position from L2 to L5. Asterisks indicate significant difference between treatments: untreated control (UTC), thinned with ethephon (ET), thinned with polysorbate 20 (P20), thinned with polysorbate 80 (P80) ( $p < 0.05$ )

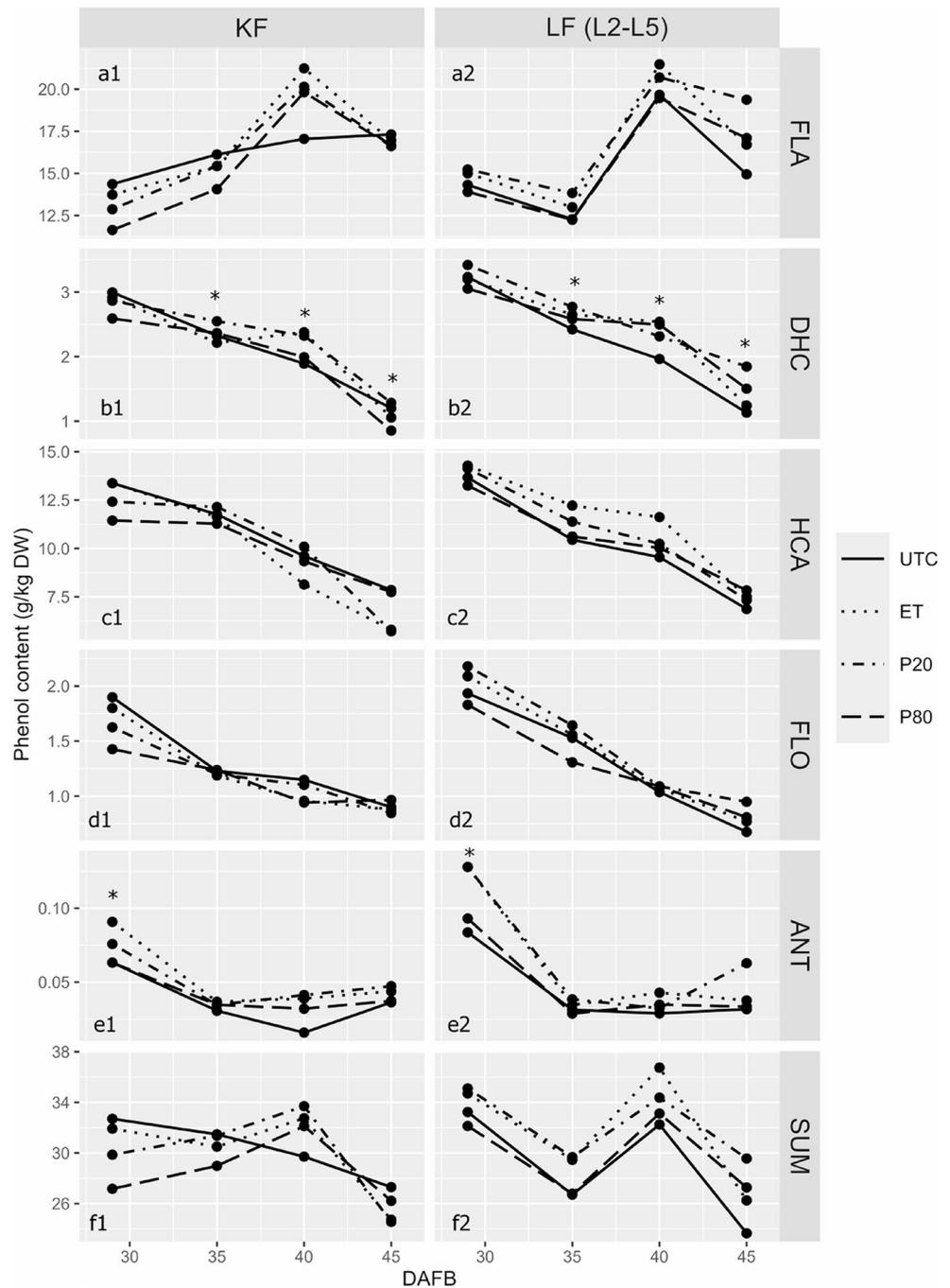


When thinning is carried out after fruitlets reach 18–20 mm in diameter, the efficacy of several standard thinning agents may decline (Verma et al. 2023); however, it has been shown that ethephon can be used at larger diameters (Marini 1996). The results of this and our previous studies (Stopar and Hladnik 2020, 2021) show that polysorbates can be applied at fruitlet diameters of 18–22 mm. Therefore, polysorbates are suitable for late or corrective thinning and may serve as a complementary tool in integrated thinning programmes.

Since the dominance of KF in the fruitlet cluster is clear, as the flower at king position is the first to bloom and it produces the largest fruit (Bangerth 1989; Botton et al. 2010), we aimed to determine whether this hierarchy is disturbed

by thinning agents. In the UTC treatment in 2023, a shift in cluster hierarchy was observed, as L1 overtook the king fruit (KF) in survival rate. L2 occupied the second position, while KF ranked third. This reordering of fruitlet dominance is likely related to the occurrence of a light spring frost in 2023. However, it is interesting to note that the KF dominance only shifted to the next higher level of the fruit hierarchy. In 2024 at UTC KF was the dominant fruitlet in the clusters, as consistently reported in the literature, where the central or king fruit exhibits greater sink strength and survival compared to lateral fruitlets (Bangerth 1989; Jakopic et al. 2015, 2016); L1 and L2 were in second place both with 41% of survived fruits. When either thinning agent was

**Fig. 9** The content of phenolics: flavanols (FLA, a); dihydrochalcones (DHC, b); hydroxycinnamic acids (HCA, c); flavonols (FLO, d); anthocyanins (ANT, e) and the sum of phenolics (SUM, f) in the apple fruitlets during the shedding process. KF, king fruitlets; LF (L2-L), lateral fruitlets, position from L2 to L5. Asterisks indicate significant difference between treatments: untreated control (UTC), thinned with ethephon (ET), thinned with polysorbate 20 (P20), thinned with polysorbate 80 (P80) ( $p < 0.05$ )



used, the dominance of KF in the fruitlet cluster was not as clear as in the UTC case for both years, since chemical thinning can alter fruitlet-specific ethylene responses, and even dominant KF may enter the abscission pathway under thinning conditions, resulting in a reduced dominance hierarchy within the cluster (Kolarič et al. 2011).

In both years, natural shedding was observed at UTC, as two groups of fruitlet drop could generally be distinguished: KF together with L1 and L2 abscised less than the L3-L5 group. However, when thinning agents were applied,

the shedding of laterals in L3-L5 group was even more expressed than at KF, L1 and L2. In addition, the diameters and fresh weights of L2-L5 fruitlets after thinning were smaller than those of L2-L5 fruitlets from UTC, indicating that L2-L5 fruitlets in thinned treatments are more likely to drop, according with results obtained by Lakso and Goffinet (2017). They concluded that the fruit drop is closely correlated with fruitlet growth, which is commonly expressed as an increase in fruitlet diameter or weight. Within the cluster, the diameter or weight of the fruitlets generally depends on

the hierarchical level. Moreover, the diameter and weight of fruitlet also depend on the sink force of the individual fruitlet, which in turn depends on the hierarchy level (Wünsche and Ferguson 2005; De Salvador et al. 2006). Overall, LF from treatments P20 and P80 had a smaller diameter or weight than UTC, indicating they are more prone to drop. This is consistent with the assumption that fruitlets with smaller diameter or slower growth exhibit lower sink strength, which reduces their ability to attract assimilates. As a result, they are less competitive within the fruit cluster and more prone to abscission (Wünsche and Ferguson 2005; Greene et al. 2013).

Sucrose was the only sugar that differed significantly between treatments. The application of P20 lowered the sucrose content in the fruitlets compared to UTC, but this was only statistically significant in the case of KF. This means that treatment with P20 slowed the accumulation of sucrose, which could partially support the carbohydrate theory for abscission. Fruitlets abscising at early stages of fruit development simply accumulate less sugar because the sink force is weaker compared to the remaining fruitlets in the cluster (Jakopic et al. 2016). This is consistent with our findings that KF treated with P20 accumulated significantly less sucrose than KF from UTC, as more fruit dropped due to the thinning effect of P20. Also in our experiment, the P20 treated KFs had a lower fruitlet weight, resulting in more fruit dropping. The same pattern of lower fruitlet weight, more fruit drop and lower sucrose content occurred in LF (L2-L5) after the application of P20 and P80, but lower sucrose accumulation was not statistically confirmed. However, Jakopic et al. (2016) found no difference in sucrose content between the central and lateral fruitlets and Stopar and Resnik (2001) also reported no differences in sucrose content between shaded fruitlets and fruitlets treated with thinning agents. Nonetheless, this does not agree with the results of Ackerman and Samach (2015), whose findings show that abscising fruitlets (L1) had higher sucrose content than surviving fruitlets (KF and L3). As abscission approaches there are clear metabolic changes in fruitlets, some of which are related to sugar metabolism, and in some cases, fruitlets about to abscise actually contain higher levels of certain sugars. This phenomenon was suggested to be a response to sugar starvation. This would mean that at an earlier point, fruitlets that will end up abscising, managed to accumulate less sucrose, while the one abscising in later stages would accumulate more sucrose before abscising (Ackerman and Samach 2015).

As sucrose is one of the most important signalling molecules (Li et al. 2012; Zhou et al. 2006), it acts as a mediator of metabolic processes and responses to environmental conditions in apple trees (Zhou et al. 2006). Sucrose can activate signalling pathways that lead to the transcription of

genes responsible for processes such as energy metabolism, growth regulation and stress responses. Sucrose can also act as a signalling molecule in response to environmental stress, helping the plant to adapt its metabolism and growth to cope with unfavourable conditions (Li et al. 2012). This could also be one explanation for why the sucrose content differed significantly between the P20 and UTC treatments, as trees treated with P20 also showed some visible stress responses. During the experiment, a mild phytotoxic effect was observed, such as leaf yellowing and slight leaf drop (data not shown), which may have affected carbohydrate metabolism.

During natural shedding, the phenolic content in the apple fruitlets decreased in all treatments and fruitlet positions, except for flavanols. The dynamics were generally similar in the KF and LF, but the response of certain phenolic groups varied significantly depending on the treatment applied. The sum of total phenolics largely reflected the flavanols dynamics due to their relative abundance, with no significant shifts between treatments or positions. This is consistent with the findings of (Jakopic et al. 2016), who observed that total phenolic content decreases during fruitlet development and correlates with fruit size and sink strength rather than treatment stress per se. In addition, Jakopic et al. (2016) and Zhang et al. (2010) reported a similar decrease in total phenolic content as observed in our study. Jakopic et al. (2016) concluded that the sharp decrease in total phenolics after 40 DAFB probably reflects increased sugar accumulation and increased metabolic activity during cell enlargement; however, our results do not show an enhance in sugar sum, but rather a stagnation of sugar during the period of cell enlargement.

Dihydrochalcones, which are known to respond to stress, decreased steadily across all investigation time points, but were significantly elevated in P20-treated fruitlets compared to UTC, particularly in the last three investigations in both the king and lateral positions. This elevated dihydrochalcones profile may reflect a stress response mediated by phenolics, which is consistent with reports linking elevated dihydrochalcones to environmental stress and risk of shedding (Dixon and Paiva 1995; Jakopic et al. 2016; Slatnar et al. 2012). Hydroxycinnamic acids and flavonols followed a similar trend of slow reduction, with no significant differences between treatments, suggesting that these compounds are associated with developmental progression rather than external thinning effects.

Anthocyanins showed a different pattern, decreasing sharply at 29 DAFB before recovering or stabilising. Fruitlets treated with ET and especially with P20 showed significantly higher anthocyanins levels compared to UTC. This could indicate a physiological response related to oxidative or phytotoxic stress. Anthocyanins are associated

with photoprotection under abiotic stress (Dixon and Paiva 1995), and their increase under P20 could be related to the potential phytotoxic effects of polysorbate treatment. In addition, Dixon and Paiva (1995) emphasised the general role of flavonoids in plant stress defence and the accumulation of phenols, especially dihydrochalcones and hydroxycinnamic acids, plays an important role in photoprotective responses under stress conditions such as solar radiation (Zupan et al. 2014). This hypothesis is also supported by the observed visual symptoms such as light yellowing and light leaf drop in the polysorbate-treated trees, which could indicate a broader stress response (data not shown). Furthermore, P20-threatened fruitlets groups KF and L2 had statistically smaller diameters, which could also be due to stress response. In addition, the differences in anthocyanins content between the P20 and P80 treatments in favour of P20 could indicate that P20 has a stronger thinning effect, which is confirmed by the larger fruit drop.

## Conclusion

The polysorbates P20 and P80 effectively induced fruitlet abscission, with both showing thinning efficiency comparable to the standard thinning agents ET and MET. Thinning treatments disrupted the natural hierarchy within the fruitlet cluster in terms of fruitlet survival, meaning fruitlets with reduced size and weight had lower sink strength, particularly in the P20 and P80 treatments, making them more prone to drop. Thinning agents affected the abscission rate within the fruitlet cluster, as the shedding dynamics and growth rate differed between the groups KF-L1 and L2-L5 in both years tested. Fruitlets in group L2-L5 were smaller in diameter and weight, as well as they had a higher fruit drop compared to KF-L1, especially when treated with thinning agents. Among sugars, sucrose was the only sugar affected by the treatment, it decreased in the P20-treated KF, which weakened their sink strength and increased abscission. As an important signalling molecule, sucrose could also mediate stress responses, further explaining the effects of the treatment. Moreover, given the specific response of certain phenolic subclasses to polysorbate treatment (particularly dihydrochalcones and anthocyanins), phenolic profiling remains a sensitive marker of stress in the early stages of fruit development and could offer insights into the physiological mechanisms underlying the thinning response. Our results partially support the carbohydrate theory of apple fruitlet abscission. In addition, the data suggest that fruit shedding may represent a form of stress, as indicated by the reduced sucrose content and increased levels of specific phenolic compounds. Further research should be conducted

to determine the exact physiological mechanisms of fruitlet abscission by which polysorbates induce a thinning effect.

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**Data Availability** The datasets generated during current study are available in the Repository of the University of Ljubljana, [20.500.12556/RUL-175240](https://doi.org/10.2500/201505/RUL-175240).

## Declarations

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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