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דילול כימי מאוחר בתפוח, מותאם לחיזוי נשירה עתידית באמצעות סמנים מולקולריים.

Late chemical thinning in Apple based on prediction of future abscission using molecular markers

מוגש לקרן המדען הראשי במשרד החקלאות

ע"י

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תקציר:

הצגת הבעיה: המידע שנאגר בעולם ובניסויים שערכנו מראה שהיום אין לחקלאי אמצעי לחזות את מידת נשירת החנטים בתפוח. לכן, מתוך זהירות, מרבית החקלאים נמנעים מדילול כימי מלא, הכולל לפי ההמלצות שני מועדי ריסוס. הדילול הידני שמבצעים לאחר הנשירה הטבעית הוא יקר ופחות יעיל. מספר סמנים גנטיים שדגם ביטויים משתנה בהתאם לפוטנציאל נשירת החנט זוהו על ידנו וישמשו כאן לחזות את מידת הנשירה. שיטות עבודה: שימוש בחומרי דילול חדשים, בדיקת השפעת הצללה, מעקב אחר דגם ביטוי סמנים אלו בחנטים, יחד עם מעקב אחר נשירת חנטים דילול חדשים, בדיקת השפעת הצללה, מעקב אחר דגם ביטוי סמנים אלו בחנטים, יחד עם מעקב אחר נשירת חנטים והתפתחות פירות כתלות בטיפולי הריסוס הכימי השונים, בזנים שונים ובאזורי גידול שונים. טיפולי דילול בשלושה אתרים בזנים זהוב וקריפס פינק, ובשני אתרים בזן טופ רד. **תוצאות עיקריות:** נמצא שהצללה בצד הדרומי של השורה מגבירה נשירה בזהום וקריפס פינק, ובשני אתרים בזן טופ רד. **תוצאות עיקריות:** נמצא שהצללה בצד הדרומי של השורה מגבירה נשירה בזהום קריפס פינק, ובשני אתרים בזן טופ רד. תוצאות עיקריות: נמצא שהצללה בצד הדרומי של השורה מגבירה נשירה בזהום קרים לבמערה בזן טופ רד. רמת הביטוי של הסמן 13/2007 בחנט מגבירה נשירה בזהום, שבזן חותה, ושטיפול מגביר נשירה בזן טופ רד. רמת הביטוים לכן מידת הנשירה בו שותה, ושטיפול מנום למצירה נשירה בזן טופ רד. רמת הביטוי של הסמן 13/2007 בחנט מגםיל בעירה נשירה עתידית מנום מנות מנות מנות מוצאות שיקיות: ביצענו את כל המשימות, תוצאות השנה השולב של 13/2007 ימים משיא פריחה מנבא מידת נשירה עתידית של חנטים. מסקנות: ביצענו את כל המשימות, תוצאות השנה השנום שנים מו שנינות, חיזקנו את הקשר בין רמת ABA התחלתית בחנטים צעירים למידת נשירה עתידית. רשמנו פטנט על שילוב החומרים החדש בטופ רד. ננסה אותו בשנה רביעית גם בזנים אחרים. נראה שניתן יהיה לנבא השנו פטנט על שילוב החומרים החדש בטופ רד. ננסה אותו בשנה רביעית גם בזנים אחרים. נראה שניתן יהיה לנבא רשינו פטנט על שילוב החומרים החדש בטופ רד. ננסה אותו בשנה רביעית גם בזנים אחרים. נראה שניתן יהיה לנבא בשירה עתידית בחנטים ביום 12 משיא פריחה באמצעות רמת ביטוישל גן מינות ביום 21 משית ביום בו. *ב*נסה אותו בשנה רביעית אות בזנים אחרים. גנית ביום גם ביום גו ביום בו ביום ביום מניתו ביום ביום ביום ביום מיב

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1. Introduction:

Abscission of young fruitlets is a widespread phenomenon in fruit trees. For some fruit crops, the rate of abscission is agriculturally sufficient, and in many cases too intense. In apples (Malus domestica) abscission is insufficient since without additional fruitlet thinning, fruits will not reach commercial size and trees will enter a cycle of alternate bearing. Manual hand thinning requires many hours of labor, thus chemical thinning agents are used for thinning. An apple inflorescence contains 5-6 flowers, the terminal 'king' flower is the first to initiate, the first to reach anthesis, and is considered the fruitlet with the lowest chance to go through abscission. The last flower to initiate and later reach anthesis is termed lateral 1 (L1), and it has the highest probability to enter abscission. Today, a farmer has no means of predicting the extent of natural fruitlet abscission in the apple

Cultivar	Orchard	Treatment	
Golden Delicious	Matityahu	Control	
		Agriton 0.40% 3 DAFB	- Brevis 0.20% 14 DAFB Brevis 0.40% 14 DAFB
	Malkia	Control	
		Agriton 0.30% 4 DAFB	- Brevis 0.20% 16 DAFB
		Control	
	Fichman	Agriton 0.40% 3 DAFB	- Brevis 0.2% 12 DAFB Brevis 0.4% 12 DAFB
Top Red	Matityahu	Control	
		Magic 2% 16 DAFB	
		Magic 2% 16 DAFB	
		Brevis 0.2% 10 DAFB	Protone 0.3% 19 DAFB Brevis 0.2% 16 DAFB
		Control	
	El Rom	Magic 2% 14 DAFB	
		Magic 2% 14 DAFB	Protone 0.3% 16 DAFB
			Protone 0.3% 16 DAFB
Cripps Pink		Control	
	Matityahu	Agriton 0.25% 7 DAFB	- Brevis 0.25% 15 DAFB Turbo 20 ppm 15 DAFB
		-	Turbo 20 ppm 15 DAFB
	Malkia	Control	
	Alfanol 6ppm+Dilamide 70ppm 5		
	El Rom	Cor	ntrol
		Agriton 0.25% 7 DAFB	- Brevis 0.25% 13 DAFB

tree, and in cases where it will be high, chemical thinning applications, only effective when applied before the end of natural thinning, can cause yield loss. Out of caution, most farmers refrain from aggressive chemical thinning, and as a result, not enough fruitlets abscise, leading to small fruit and alternate bearing.

- 2. Research Aims: are to answer the following research questions:
- Is total apple abscission reduced in a year in which internal abscission processes in L1 fruitlets initialized earlier?
- Can we predict natural apple fruitlet abscission and chemical-derived abscission by analyzing samples of fruitlets 8-12 days after full bloom?
- Can we provide farmers with a prediction of future abscission at a time in which he can still intervene successfully with additional chemical treatments?

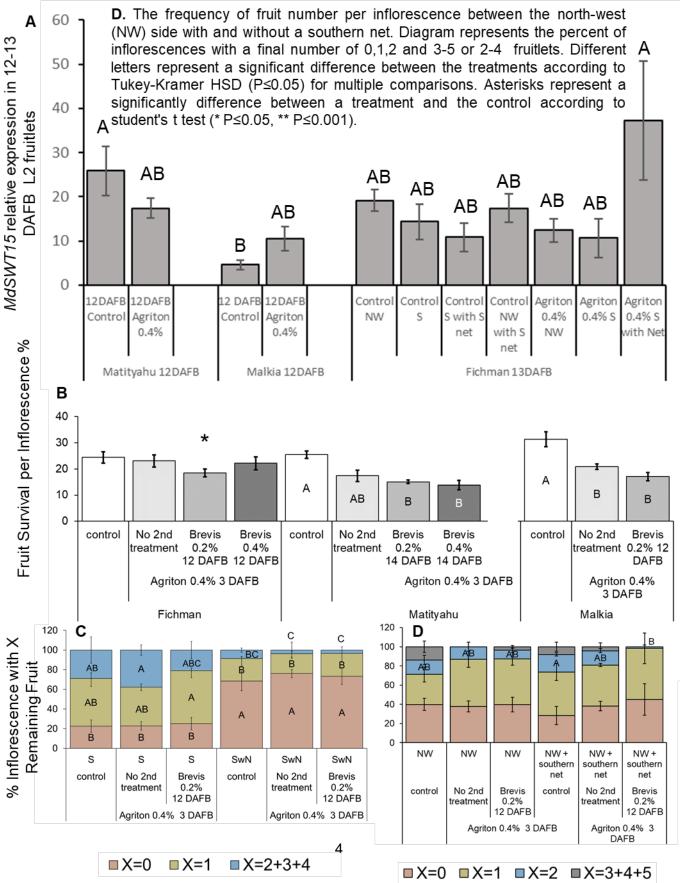
 Can molecular markers initially identified in 'Golden Delicious' also predict of abscission fate in cultivars 'TopRed' and 'Cripps Pink'? Will variability in growing regions or horticultural treatments have an effect on the correlation between marker gene expression and future abscission?

• 3. Detailed description of main experiments and research results:

Experiments were conducted in 8 orchard/Cultivar combinations, altogether 32 treatments (Table 1), each treatment 5 trees. In each experiment we collected data on L2 fruitlet gene expression, final fruitlet number after abscission, fruit number per tree at harvest, final fruit weight per tree, and fruit size distribution. Following year flowering will be measured and calculated by mid-May 2021. In addition, we measured hormone levels in specific fruit, and treated 'Golden Delicious' trees at Fichman to an additional treatment of netting on the south side. Unfortunately, it is impossible to squeeze text and all graphs within 10 pages, thus some results are mentioned and not presented in this report.

3.1' Golden Delicious' trials were conducted in three orchards (Table 1). This spring the trees in Fichman had much fewer inflorescences per tree than those in Matityahu. Still, the natural survival rate of fruitlets in untreated trees was similar in Matityahu and Fichman (~24-25%) and higher in Malkia (~31%) (Fig. 1B). In these control trees, the frequency of inflorescences with 0 or 1 final fruitlets was 67-70% in Fichman and Matityahu, and only ~49% in Malkia, since inflorescences in Malkia retained additional fruitlets. With similar abscission rates and much more flowering in Matityahu, the average final fruit number per control tree was much higher in Matityahu (736) compared to Fichman (394). The percent of fruits with a diameter of 70 or greater in control trees was 13% in Matityahu and 16.7% in Fichman. Thus, fruit size seemed to be less affected by overall fruit number in the tree and more effected by the number of fruitlets per inflorescence. MdSWEET15 levels in L2 fruitlets of control trees was lower in Malkia compared to Matityahu and Fichman (Fig. 1A), and this correlated well with lower fruitlet abscission in Malkia (Fig. 1B). Only in Malkia did the Agriton treatment by itself cause a significant reduction in survival, compared to non-treated control trees (Fig. 1B). Accordingly, Malkia was the only orchard in which the Agriton treatment caused an increase (though not statistically significant) in *MdSWEET15* expression compared to control (Fig. 1A). In line with the above findings, Malkia was the only orchard where Agriton significantly increased the percent of inflorescences with 0-1 remaining fruitlets, decreasing the percent of inflorescences with 2-3 remaining fruitlets. The Agriton treatment significantly increased the percent of fruit with a diameter of at least 75mm in Fichman.

Fig. 1. 'Golden Delicious' fruitlet gene expression, survival and fruitlet distribution in response to chemical thinning in the spring of 2020. A. *MdSWEET15* relative expression, measured by quantitative real-time PCR, using histone H3 (MDP0000263445) as a house keeping gene, in L2' fruitlets in response to early chemical thinners. **B.** Survival rate was assessed at the end of natural abscission on 26 pre-marked inflorescences per tree. Numbers are mean values of independent biological repeats (5 trees). Trees were either sprayed or not sprayed with 0.4% Agriton (Naphthaleneacetic acid (NAA) and 1Naphthaleneacetamide-(NAD)) at 3-6 Days after full bloom (DAFB). And then further sprayed or not sprayed with 0.2-0.4% Brevis (metamitron) at at 12-14 DAFB. **C**. The frequency of fruit number per inflorescence between the south (S) side with and without a net.



Later 0.2% Brevis (Metamitron) treatments, following Agriton treatment, significantly reduced fruitlet survival in Fichman and Matityahu compared to control trees (Fig. 1B), yet did not significantly increase abscission in Malkia, in which the Agriton treatment alone had an effect (Fig 1B). Still, in Malkia the frequency of inflorescences with 2 or 3 fruitlets was significantly reduced by the 0.2% Brevis treatment. The frequency of inflorescences with 0 or 1 fruitlets was significantly increased by the 0.2% Brevis treatment in Fichman (Fig 1CD) and not in other orchards or concentrations. Final fruit number in Matityahu was significantly reduced by the Brevis 0.2% treatment, compared to control trees. This caused the percent of fruit with a diameter of at least 70mm to increase from 13% in control trees to 53.4% in this treatment in Matityahu. Brevis treatment, compared to Agriton alone, also increased the percent of fruit with a diameter of 70 mm in Fichman. Last year, in Fichman Golden Delicious we noticed higher fruit survival at the side of the tree facing South East, compared to North West. Since we suspected that this was caused by higher irradiance on the south east side, we added this year an additional set of trees in which we added two layers of 40% shading net on the south side (one quarter) of the tree. We added the net after flowering, not to interfere with pollination, on April 23rd, 6 DAFB. Nets were removed 53 days later. When comparing the south side, with or without net, the net caused a significant increase in inflorescences with 0 remaining fruit (Fig 1C) in all treatments. The net was applied 1 week before fruitlet sampling. The effect of the net on MdSWEET15 levels in L2 fruitlets expression was apparent within a week in trees treated with Agriton at 3 DAFB (Fig 1A). On the Northwest side of the tree, the presence of nets on the south side of the tree had no significant effect on fruitlet survival (Fig 1D). The chemical thinning treatments had little effect on fruitlet survival, compared to the net application (Fig 1CD).

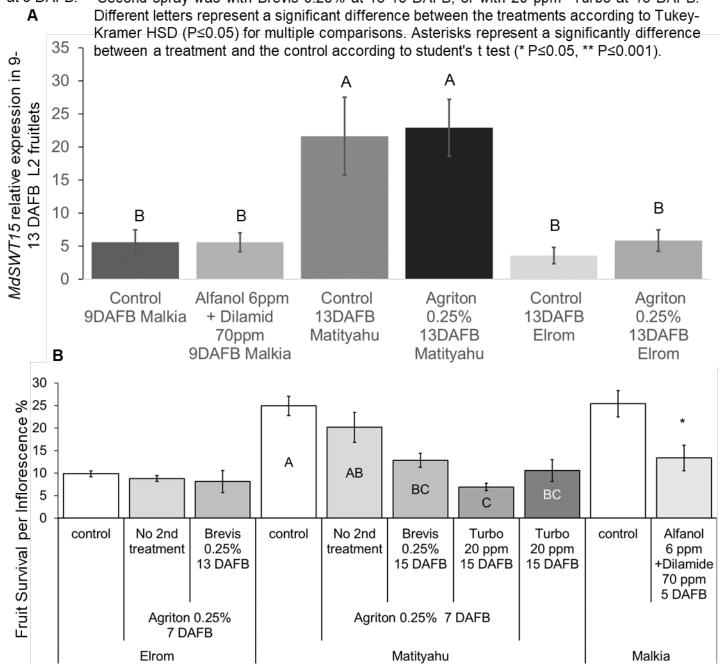
3.2' Cripps Pink'. This year, experiments were conducted in Matityahu and Malkia in the Galilee, and Elrom in the Golan Heights. At Malkia, the trees were relatively small, with an amount of inflorescences that matches their size. At Elrom, the flowering rate was good. However, low temperatures in the first days after full bloom might have affected pollination later reducing fruit set. Additionally, the trees in the plot had powdery mildew. Likely these conditions contributed to the low natural survival rate (~10%) in the control Cripps pink trees of Elrom (Fig. 2B) compared to those in Matityahu and Malkia (~25%; Fig. 2B). As a consequence, the frequency of inflorescences with 0 or 1 remaining fruitlets was ~91% in control trees of Elrom, and 65-67% in Matityahu and Malkia. Fruit number and diameter was not measured in Malkia. The average final fruit number per tree of untreated trees was ~202 in Elrom and ~760 in Matityahu. This small number of fruits in Elrom caused an increase in fruit size with fruits of at least 75mm reaching ~50% in Elrom and only 10.6% in Matityahu. *MdSWEET15* levels in L2 fruitlets of untreated Cripps Pink trees did not provide an accurate estimate of further abscission when comparing locations (Fig. 8B). Relatively low levels in Malkia are likely due to a mistake in sampling date, as fruitlets were collected 4 days earlier (9 DAFB). Relatively low levels in Elrom at 13 DAFB did not match the high rates of abscission later

detected. It is likely that in conditions of low pollination or later on (after sampling) disease (powdery

mildew), levels of *MdSWEET15* in developing L2 fruitlets do not correlate well with final fruitlet

numbers.

Fig. 2. 'Cripps Pink' fruitlet survival in response to chemical thinning and return bloom in the spring of 2020. A. *MdSWEET15* relative expression, measured by quantitative real-time PCR, using histone H3 (MDP0000263445) as a house keeping gene, in L2' fruitlets in response to early chemical thinners. **B.** Survival rate was assessed at the end of natural abscission on 26 pre-marked inflorescences per tree. Numbers are mean values of independent biological repeats (5 trees). Trees were either sprayed or not sprayed with Agriton 0.25% at 7 DAFB or with a combination of with 6 ppm Alfanol (20 ppm NAPHTHALENE ACETIC ACID (NAA)) and 70 ppm Dilamide (10% NAPHTHALENE ACETAMIDE (NAD)) at 5 DAFB. Second spray was with Brevis 0.25% at 13-15 DAFB, or with 20 ppm Turbo at 15 DAFB.



The single Agriton treatments in Elrom and Matityahu had no significant effect on expression of *MdSWEET15* in the L2 fruitlets (Fig 2A) overall fruitlet survival (Fig. 2B) and fruitlet distribution. In Malkia, the Agriton treatment was replaced by a single Alfanol(6ppm)+Dilamide(70ppm) treatment

at 5 DAFB. Compared to untreated trees, this treatment significantly reduced fruitlet survival (Fig. 2B), and significantly increased the percent of inflorescences with 0-1 fruitlets. Fruitlet samples taken only 2 days after the thinning treatment did not detect a significant increase in *MdSWEET15* in L2 fruitlets (Fig. 2A). Perhaps it takes longer for changes in gene expression to become apparent after chemical thinning.

In Matityahu, Turbo treatment at 15 DAFB without or with previous Agriton treatment caused a significant reduction in surviving fruitlets compared to control trees (Fig. 2B). The treatments also significantly increased the percent of inflorescences with 0-1 remaining fruitlets. There is an advantage for using a single Turbo treatment at 15DAFB or slightly later since it seems to be efficient, and may be given after analyzing data on gene expression at an earlier time point.

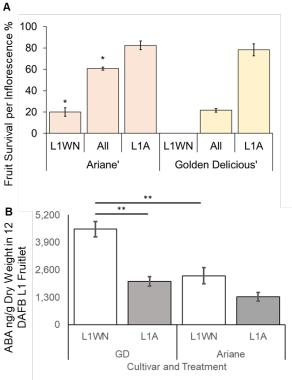
Brevis treatments were given in Elrom and Matityahu. In Elrom, where abscission was very high in control trees, the combined treatment of Agriton + Brevis (at a later date) did not increase abscission (Fig. 2B), did not increase the percent of inflorescences with 0-1 fruitlets, did not significantly reduce fruit numbers or increase fruit diameter. In Matityahu, the combination of Agriton followed by Brevis caused a significant reduction in fruitlet survival compared to control trees (Fig. 2B), followed by a significant increase in percentage of inflorescences with 0-1 remaining fruitlets, a significant decrease in final fruit numbers per tree, and a significant increase in fruits with a diameter of at least 70 mm. This is a nice example in which the combination of Agriton followed by Brevis provided significant thinning in the orchard which required thinning (Matityahu) and had no effect in the farm that did not require thinning (Elrom). Still, the single treatment with Turbo at 15 DAFB was just as effective in all parameters.

3.3 Comparing 'Ariane' and 'Golden Delicious' Fruitlet natural abscission in the 'Ariane' cultivar is much lower compared to Golden Delicious [1]. We asked whether L1 fruitlet survival in 'Ariane' is influenced by neighbors. As expected from previous results, total fruitlet survival in Ariane (60%) was much higher than in neighboring Golden delicious trees of the same age (~20%; Fig. 3A). L1 fruitlet natural survival rate in 'Ariane' reached 20%, and 0% in Golden Delicious (Fig. 3A). Previously it was shown that L1 survival in Golden Delicious increased when all neighbors were removed [1]. Removing all other fruit in the inflorescence at full bloom, increased the survival rate of the L1 fruitlet (L1 Alone; L1A) to 78% in Golden Delicious and 82% in Ariane (Fig. 13A). Thus, without neighbors, the survival rate of L1A was similar in both cultivars (Fig. 3A). This might suggest that the difference between these cultivars is not within L1 fruitlets but depends on how L1 fruitlets react to neighboring fruit. We wanted to compare the endogenous levels of ABA in the L1 fruitlet of each cultivar, given the presence of neighbors in the inflorescence. The main objective is to test whether the lower levels

of natural abscission in 'Ariane' correlate with

lower levels of ABA in 'Ariane' fruitlets than 'Golden Delicious' fruitlets. For the experiment, L1A/WN fruitlets from both cultivars sampled 12DAFB. The were hormonal measurement was performed as a paid service at the Volcani Institute.

Indeed when comparing L1WN fruitlets from 'Golden Delicious' and 'Ariane' the endogenous ABA levels in 'Ariane' were significantly Fig. 3. Comparing 'Golden Delicious' 'Ariane' to fruitlet survival and ABA internal levels in L1 fruitlets with or without neighboring fruits. Α. Average percent survival of L1 fruitlets, with (L1WN) or without (L1A) neighbors, and of all fruitlets in the inflorescence. B. ABA levels (calculated for 1 gram of dry weight) in 12 DAFB L1 fruitlets Asterisks in A represent а significantly difference between the two cultivars in the same treatment according to student's t test. Asterisks in В compare between cultivars in L1WN and within Golden Delicious comparing L1 fruitlets with or without neighbors (* P≤0.05, P≤0.001).



lower (Fig. 3B). When comparing the effect of neighboring fruit removal, it caused a reduction in ABA levels

of L1 fruitlets in both cultivars, with the difference in 'Golden Delicious' being significant. (Fig. 3B). These results put a spotlight on ABA levels as possible early triggers of fruitlet abscission, since levels of ABA at 12 DAFB nicely correlate with later on abscission, and explain why certain cultivars (Ariane) tend not to abscise many fruitlets. These results confirm previous findings that applying endogenous ABA, currently sold as S-ABA ('Protone') should be effective as a chemical thinner in apples[2].

3.4 'Top Red'. A successful chemical thinning protocol for 'Top Red' is still unavailable. Without strong thinning, this cultivar can be strongly alternate bearing [3]. The only current solution in this cultivar is expensive hand thinning, leaving some inflorescences with 0 fruitlets, and others with 1 fruitlet. The use of S-ABA may be efficient yet the commercial product is relatively expensive. Previous research in our lab led to a hypothesis that pre-treating trees with Magic (Uniconazole P) would allow effective fruitlet thinning by Protone (s-ABA) at low concentrations [4]. This was due to the fact that uniconazole P, in addition to inhibiting Gibberellin biosynthesis, is also an inhibitor of ABA degradation [5]. Additionally, results from our experiments in previous years suggest that Uniconazole P treatment can cause abscission of apple fruitlets mostly by inhibiting ABA catabolism. We hypothesized that relatively high levels of Protone are required for apple thinning since a large portion of the external ABA is efficiently catabolized within the plant on arrival, thus reducing the treatment efficiency [4]. Pretreating the trees with uniconazole would inhibit this catabolism, allowing lower concentrations of Protone to be more effective. To evaluate the combined treatment's

effectiveness, as a control we sprayed other trees in the same rows with just one of the chemicals or none of the chemicals. We tried these treatments in both Matityahu and Elrom (Fig 4). Control tree fruitlet survival rate was ~28% at Matitiyahu and only ~11% at Elrom (Fig. 4B). This low survival rate in Elrom was likely due to weather conditions in this year in the Golan heights since similar levels were reported in other orchards in the region. Control inflorescences with 0 or 1 remaining fruit reached ~98% at Elrom and~44% in Matityahu. Clearly, in retrospect the orchard at Elrom did not require additional chemical

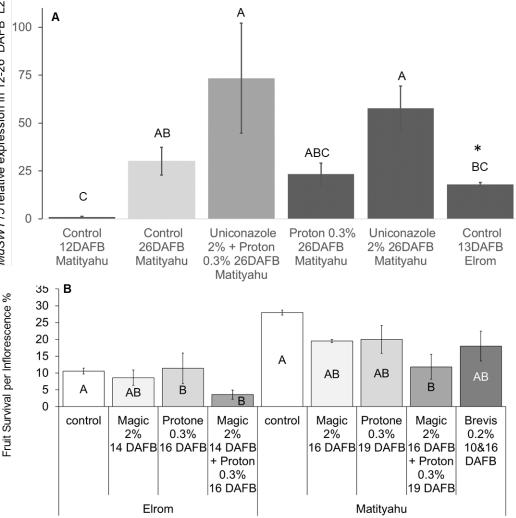
fruitlets

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DAFB

MdSWT15 relative expression in 12-26

Fig. 4. 'Top Red' fruitlet expression and survival in response to chemical thinning. A. MdSWEET15 relative expression, measured by quantitative real-time PCR, using histone H3 (MDP0000263445) as a house keeping gene, in L2' fruitlets in response to chemical thinners. B. Survival rate was assessed at the end of natural abscission on 26 pre-marked inflorescences per tree. Numbers are mean values of independent biological repeats (5 trees). Trees were either sprayed or not sprayed with 2% Magic (Inhibitor of GA biosynthesis contain 50 g/L Uniconazole) or with 0.3% Protone (SG 20% S-ABA) at 16-19 DAFB. Trees sprayed with Uniconazole 2% were further sprayed with Proton 0.3% at 16-19 DAFB. Trees were sprayed with Brevis 0.2% at 10 DAFB or then were further sprayed with Brevis 0.2% at 16 DAFB. Orchards were in Elrom and Matityahu. Different letters represent a significant difference between the treatments according to Tukey-Kramer HSD (P≤0.05) for multiple comparisons. The Asterisk in A represent a significant difference between control trees in Elrom and Matitvahu according to student's t test (P≤0.05).



thinning. This could have been obvious by studying expression of MdSWEET15 in control trees of both orchards (Fig. 4A). If we would have managed to obtain these results before spraying we could have avoided chemical thinning at Elrom. At both orchards, the combined treatment with Uniconazole P followed by S-ABA caused a significant decrease in fruitlet survival, while single treatments had no significant effect (Fig. 4B). At Elrom, the percent of control inflorescences with 0-1 remaining fruitlets was already 98%, so the combined treatment did not significantly increase this percentage. At Matityahu, the percent of control inflorescences with 0-1 remaining fruitlets was significantly increased by the double treatment. Total fruit number was reduced from 466 in control to 280 in the combined treatment in Matityahu. This decreased the percent of fruit below 70mm from 60% to 44% .

We sampled fruitlets at 26 DAFB to test the effect of the treatments at 16-19 DAFB on gene expression. The expression of *MdSWEET15* in 26 DAFB fruitlets of trees that received uniconazole with or with S-ABA was higher, though not significantly, compared to control trees of the same age (Fig. 4A). At Matityahu, apart from the Magic and Protone treatments, trees were treated twice with Brevis 0.2%, at 10 and 14 DAFB. The survival rate was lower than controls, yet the decrease was not statistically significant (Fig. 4B).

4.Discussion:

We accomplished all the experiments that we were committed to, and added additional experiments. The effect of Agriton followed by Brevis was significant compared to untreated trees of Golden Delicious. The contribution of the Brevis treatment to overall thinning was not always significant. Adding shade nets on the south side of trees caused significant thinning. Shading and Brevis might function in a similar way, reducing carbohydrate levels available to the fruitlets. It is not clear at this time if shading is a practical (better for the environment) replacement for chemical thinning. This should be studied further on in a separate study. Expression of the MdSWEET15 gene as a predicter of further abscission seemed to work in some but not all cases. Cases in which it could not predict correctly were the Cripps Pink control trees in Elrom, in which low pollination and later disease caused low fruitlet survival which was not predicted by gene expression. In addition, collecting tissues to early (Cripps Pink Malkia) does not provide useful data for comparison. MdSWEET 15 levels did predict several other situations here. We strengthened our knowledge on the internal role of ABA in natural thinning and we developed a new protocol, which worked successfully, to thin Top Red by combining Uniconazole P and Protone. Different concentrations and timing will be tested next year in Top Red, and the efficacy of this treatment will be also trialed in other cultivars.

Publications and Talks:

A. Samach, M. Ackerman-Lavert, P. Fresnillo, O. Craine. 2020, Use of uniconazole for potentiating abscisic acid effects in plants. US Provisional Patent Application No 63/077,626., 2020.

A. Samach (2021) Practical insights from the study of early molecular events in natural apple fruitlet abscission. Invited Speaker at EUFRIN WG Thinning 2021 online meeting, February 25-26th, PCfruit, Sint-Truiden, Belgium.

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