Project Title: Finding peel surface properties linked with green spot of WA 38

Report Type: Final Project Report

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Budget 1

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Item	2024
Salaries	
Benefits	
Wages	
Benefits	
RCA Room Rental	
Shipping	
Supplies	$4,000.00
Travel	
Plot Fees	
Miscellaneous	
Total	$4,000.00

RE-CAP OBJECTIVES:

1. Cuticle deposition during development in WA 38 and the parent cultivars Honeycrisp and Enterprise. Fruit growth will be monitored.

2. Development of lenticels and their extent of opening. Cross-sections of the fruit's skin will be taken to monitor the development of epidermis and hypodermis in symptomatic and asymptomatic fruit.

SIGNIFICANT FINDINGS:

1. ***Cuticle deposition during development in 'WA 38' and the parent cultivars 'Honeycrisp' and 'Enterprise'.***
   - A new stage of "WA 38" green spot development was identified during the sampling of the cuticle throughout the season. We named "green halo" first observed on 6/4/2024 = 47 DAFB.
   - Across 18 weeks, we followed the apple growth and noticed some differences among varieties.

2. Development of lenticels and their extent of opening.

- Fruit growth in the green spot region (GS) is inhibited, as indicated by slight depressions of the fruit surface in those regions where green spots form. This confirms the results of the previous project, and GS seems to be likely linked to the lenticel damage.

- Bags confirmed the results from previous studies. Early bagged fruit did not develop signs of "Severe" green spot, while the control showed 29.7 % of “Severe” symptoms (culled apples).
- No significant differences were found in the incidence of GS within each bearing wood.
- Lenticels in “bag” apples presented a higher AO infiltrated area than the "control" ones on 98 and 155 DAFB.

RESULTS AND DISCUSSION

1. Cuticle deposition during development in ‘WA 38’ and the parent cultivars ‘Honeycrisp’ and ‘Enterprise’.

During season 2024, we focused on investigating the cuticle deposition throughout fruit development for 'WA 38' and the parents, 'Enterprise' and 'Honeycrisp'. On 5/20/2024 (32 days after full bloom =DAFB for 'WA 38' and 'Honeycrisp', and 37 DAFB for 'Enterprise'), we started the fruitlets sampling of all 3 cultivars; the selection criteria accounted for both east and west side of the trees, all bearing positions in the canopy, and both king and lateral types of fruit. For 'WA 38', the sampling was weekly from 5/20/2024 to 7/30/2024, then became biweekly (twice a month), while for 'Enterprise' and 'Honeycrisp', was done twice monthly.

Once in the laboratory, apples were photographed on graph paper, weighted and described for any misshapenness, blush, presence/absence of green spot for every timepoint. Epidermal peel disks (8 to 10 mm punch, depending on the stage of development) were excised from each apple in the equatorial area (2 disks/apples up to 7/2/24 = 75 DAFB, then 3 disks/apples until harvest on 9/25/2024 = 160 DAFB) and placed into scintillation vials with an enzyme solution containing both pectinase and cellulase, sodium azide and citric acid as described in Lai et al., 2016 and kept at room temperature until further analysis. The punctured apples were saved and subjected to allometric measurements (included maximum diameter) to define the growth of the different areas of the fruit along the season (Skene, 1966).

For the three cultivars, we built calibration curves utilizing the "detached" apples for the cuticle deposition sampling, where fruit weight resulted in a polynomial function of the apple diameter. The R^2 were 0.9871, 0.9812, and 0.9885 for 'Enterprise', 'Honeycrisp', and 'WA 38' (Figure 1), respectively.

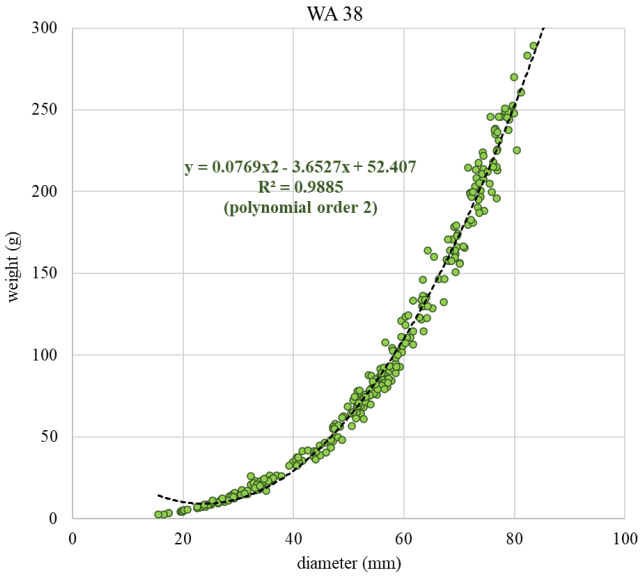

Figure 1: *'WA 38' relationship between fruit weight and its diameter for data collected in 2024 at the Sunrise Farm (WSU).*

At the first measurement on 5/20/2024, when the fruit diameter range across the 3 cultivars was 15 to 26 mm, another set of 20 east-facing apples per cultivar were tagged "on-tree" (Figure 2) and photographed weekly with the same angle settings to track the fruit growth. Post-processing the fruit maximum diameter measured from the images (N=10 best apple captures) allowed us to quantify the apple growth and present it as millimeters per day (mm/day, Figure 3). The growth chart presents weekly fruit development as each cultivar's growth speed. This parameter was compared across varieties to assess the hypothesis that there could be a difference in 'WA 38' growth compared to the two parents.

Figure 2: *'WA 38' and its parents pictured on 7/9/2024 in the weekly photographs to track the fruit dimensions and growth in 2024 at the Sunrise Farm (WSU).*

Across 18 weeks, the apple growth showed some differences that we are investigating (Figure 3). During the sampling for the cuticle development throughout the season, we were able to identify a new stage for 'WA 38' green spot development that we named "green halo" first observed on 6/4/2024 = 47 DAFB (avg. 31 mm max diameter) and characterized by a darker green ring around lenticels mainly in the shoulder and equatorial area of the apples (not shown).

Another experiment was carried out in 2024 to quantify the stress and strain relaxation by apple region and developmental stage for all three cultivars. It is well known in literature that, during their development, apples undergo a series of stress events that cause strain of their cuticle (Knoche and Lang, 2017, Knoche et al., 2018). The hypothesis behind this experiment was that the green spot (GS) onset could be related to a "skin strain event" during the apple development that causes irreversible damage to the cuticle, potentially impacting skin and cortex integrity and jeopardizing marketability. The reason for investigating by region relies upon the fact that GS is more frequent in the shoulder and equatorial areas of the apple than in the calyx. The first timepoint was at approximately 40 mm apple diameter (6/18/2024 = 61 DAFB); at that time, one 8-mm-disk of cuticle per each region was excised from each apple (blushed side) and placed into the enzyme solution described above; the three regions in the apple were "shoulder (S)", "equator (E)", and "calyx (C)". The total fruit per sampling point were 25. For the second time point, at approximately 55 mm fruit size (7/16/2024 = 89 DAFB), the number of regions of interest increased from 3 to 5. In fact, intermediate regions were included and named "shoulder-to-equator (S-E)" and "equator-to-calyx (E-C)". Sampling was completed "at harvest" for each cultivar: 9/10/24 for 'Honeycrisp' (145 DAFB) and 9/24/24 for 'WA 38' and 'Enterprise' (159 DAFB for 'WA 38'). Those ongoing biophysical analyses can eventually provide valuable insights into the cuticle properties of the three cultivars under study and their potential role in GS onset.

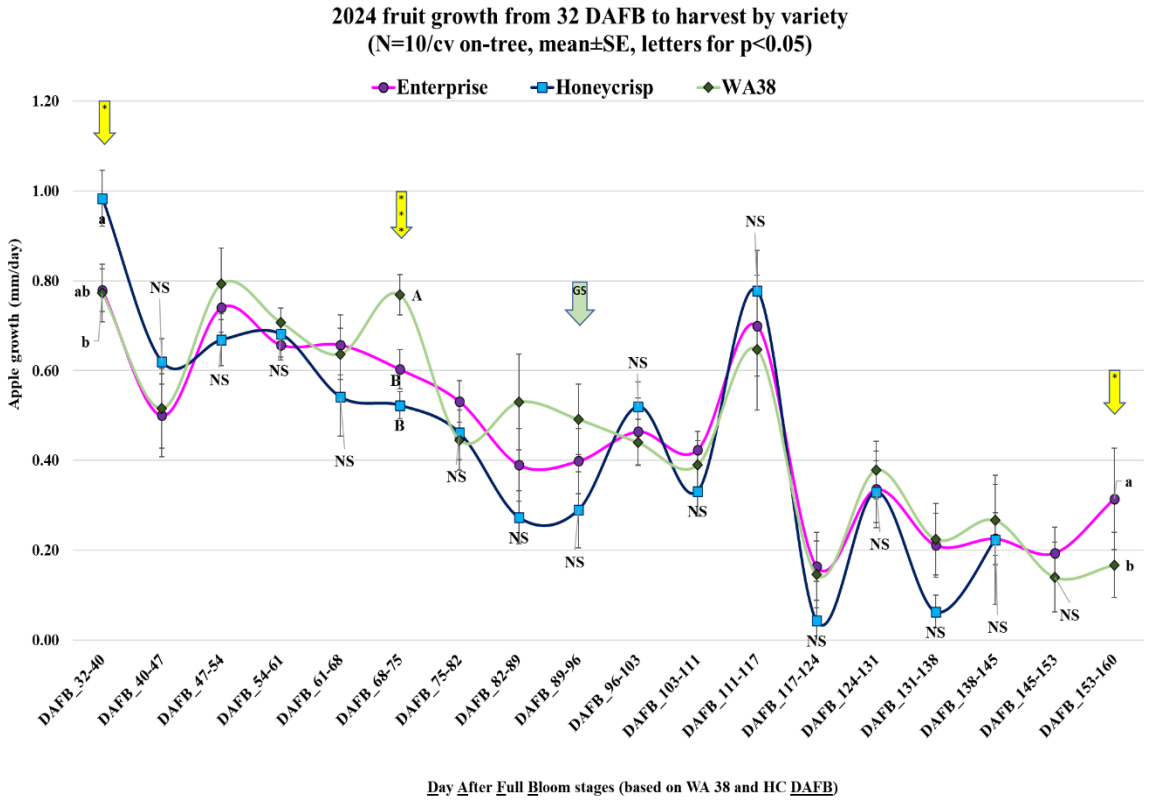

Figure 3: *'WA 38', 'Enterprise', 'Honeycrisp' on trees apples photographed weekly from May to September 2024 at the Sunrise Farm (WSU). The maximum fruit diameter measured from the images (N=10 best apple captured) was used to calculate the apple growth (millimeter per day = mm/day). The three yellow arrows pointing down indicate the 3 weeks in the season when the apple growth between the three cultivars resulted significantly different. Significance: * <0.05, *** <0.001, NS = not significant; same letter associate to the means indicate no difference. The green arrow marked with "GS" indicated the onset of green spot on 'WA 38' in 2024 in the experimental site. Error bars represent the standard error of the means.*

2. Development of lenticels and their extent of opening. Cross-sections of the fruit's skin will be taken to monitor the development of epidermis and hypodermis in symptomatic and asymptomatic fruit.

Preliminary results on 'WA 38' symptomatic and asymptomatic apples in 2023 provided some leading clues that helped direct our research for 2024. Fruit growth in the green spot region (GS) is inhibited, as indexed by slight depressions of the fruit surface in those regions where green spots form. Preliminary data indicates that cuticle mass is higher within these green spot regions (22.4 ± 0.3 g m^{-2}) as compared to non-affected control areas (20.3 ± 0.2 g m^{-2}). This is primarily due to increased wax deposition (9.0 ± 0.2 within GS vs. 6.9 ± 0.2 g m^{-2} control), while cutin deposition appears unaffected (13.4 ± 0.2 within GS vs. 13.4 ± 0.2 g m^{-2} control). Non-affected 'WA 38' fruit skin does not show any irregularities in cutin or wax deposition compared to other cultivars. The mass of cuticle per unit area in these non-affected areas is within the range normally encountered in apple varieties (range 20.3 ± 0.3 to 36.2 ± 0.9 g m^{-2}; grand mean of 22 apple varieties 26.7 ± 0.3 g m^{-2}; Khanal et al., 2013). The

occurrence of cracks in severe green spot symptomatic peel is indicative of increased mechanical stress. This is consistent with increased strain release upon wax extraction in the green spot affected area. That wax inhibits strain relaxation, a typical strategy in apple to avoid surface defects.

In Sheick et al., 2022, we had already observed that 'WA 38' lenticels were frequently at the center of the green spot symptoms, but not always. Lenticels are reported to be "weak points for mechanical stress as fruit enlarges," as reported by Duríc et al., 2015. In this objective, we focused on the development of lenticel and its involvement in the evolution of GS. On 6/5/2024, twenty 'WA 38'/G41 trees trained to spindle were selected for a similar amount of fruit and vigor by visual estimation. Ten of those twenty trees (randomized across 5 orchard rows) were tagged as "bag" trees and each cluster was hand thinned down to the strongest fruitlets and then bagged as described in Sheick et al., 2022. For each "bag" tree, at least 5 apples were left outside bags to account for an "internal control" and the average of bags/tree was 61 ranging from 47 to 95 bag/tree. The other ten trees represented the "control" and were handled by hand thinning like the "bag" trees, leaving only the strongest fruitlet in each cluster. The average apples/tree at that time was 66, ranging from 38 to 103 apples/tree across the 10 trees. During the growing season, we utilized those trees as source of fruit for sampling at 3 key developmental timepoints (7/3/24 = 76 DAFB = 50-55 mm, 7/25/2024 = 98 DAFB = 58-60 mm, and 9/20/24 at harvest = 155 DAFB = >73 mm) to compare lenticels development and microcracking between "bag" apples and "control" apples after 10-min-staining in 0.1 % (w/v) aqueous Acridine Orange (AO) + 0.05% Silwett® L-77 solution. Ten-mm-stained disks were visualized at the Nikon SMZ18 stereomicroscope under visible light and under incident fluorescence light (using a P2-EFLC EGFP LP HC filter, emission 515-555 nm) and photographed to calculate the lenticel density and the AO infiltrated area as mm^2 per lenticel (Figure 4). This experiment can make us postulate that there is possibly less mechanical stress in the skin of bagged fruit (for unknown reasons) and less need for wax deposition (data not shown).

At harvest (9/23/2024), three "bag" trees and three "control" trees were picked, keeping apples separated accordingly to the different bearing woods and classifying them as "spur", "brindilla", and "ramo misto". The apples were stored at 34 °F and graded approximately one month after harvest; data are reported as presented in Sheick et al., 2022 (Figure 5). When comparing the apples harvested from "bag" trees with those from the "control" trees, we confirmed the results from previous studies (Sheick et al., 2022) where apples, if bagged early enough in the season (beginning of June), did not develop any signs of "Severe" green spot (green spot stages 3, 4 or 5 that leads to unmarketable apples). On the other hand, the 29.7 % of the "control" apples developed severe symptoms (culled apples).

When looking at the incidence of "Mild" green spot (green spot 1, 2, or 6 affecting below 5% of apple surface) between "bag" and "control" apples, the former presented just a green halo (GH) around the lenticels (classified as green spot stage 1) in 30.1 % of the apples, while the latter developed higher GS incidence of GS equal to 62.5 % of graded apples (Figure 5). The presence of GH in the "bag" apples can be explained by the fact we observed the onset of GH around lenticels on 6/4/2024 before carrying out the bagging procedure for the whole experiment; therefore, we can state that GH did not evolve in any possible worsen stages once apples were bagged for over 15 weeks. We noticed that green spots symptoms appeared earlier in 2024, and this can explain why some GH has been observed in bagged fruit, too.

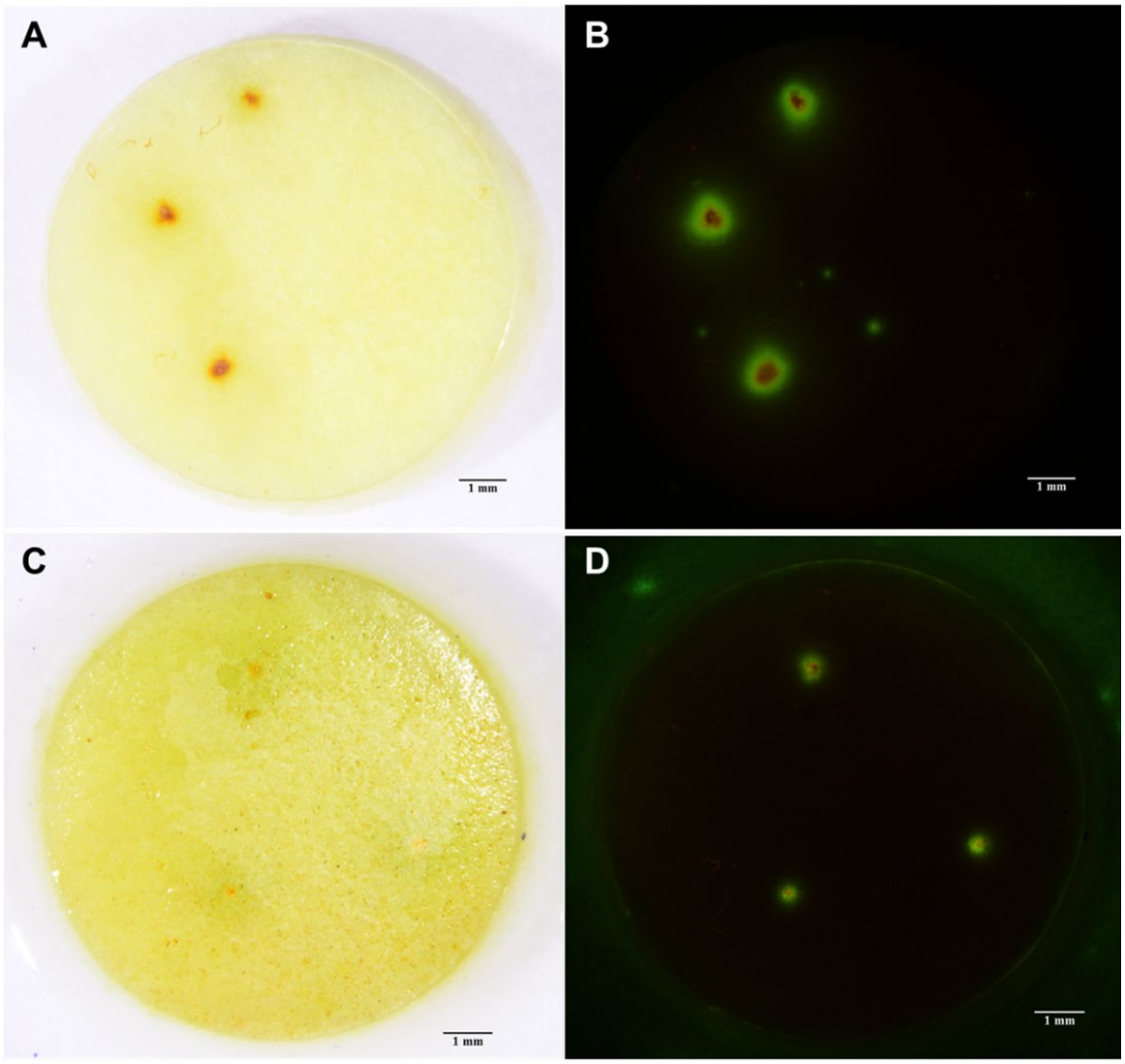

Figure 4: *Visible and fluorescent disks (epidermis) after Acridine orange (AO) staining at timepoint 2 (07/25/2024) of "bag" (A and B) and "control" (C and D) 'WA 38' apples. Disks are 10 mm in diameter, the magnification of the microscope was 0.75X for all the images. Analog gain used was 1.0X for visible (no fluorescence) and 14.0X for fluorescence light (green) both with an exposure of 30ms. (A) 10 mm disk of "bag" fruit after AO staining in visible light, and (B) in emission fluorescence green light showing the respective AO infiltrated lenticels. (C)10 mm disk of "control" fruit after AO staining in visible light, and (D) in fluorescence (green) light showing the respective AO infiltrated lenticels. White and black scale bars represent 1 mm in each respective image.*

The 2024 graded production (left on trees after the abovementioned samplings) was distributed mainly on spurs (84.5% in the "control" trees and 88.7% in the "bag" trees), followed by brindilla (11.7% in the "control" trees and 6.3% in the "bag") and ramo misto (3.8% in the "control" trees and 5% in the "bag" trees). No significant differences were found in the incidence of GS within each type of bearing wood, nor were any specific trends observed (data not shown).

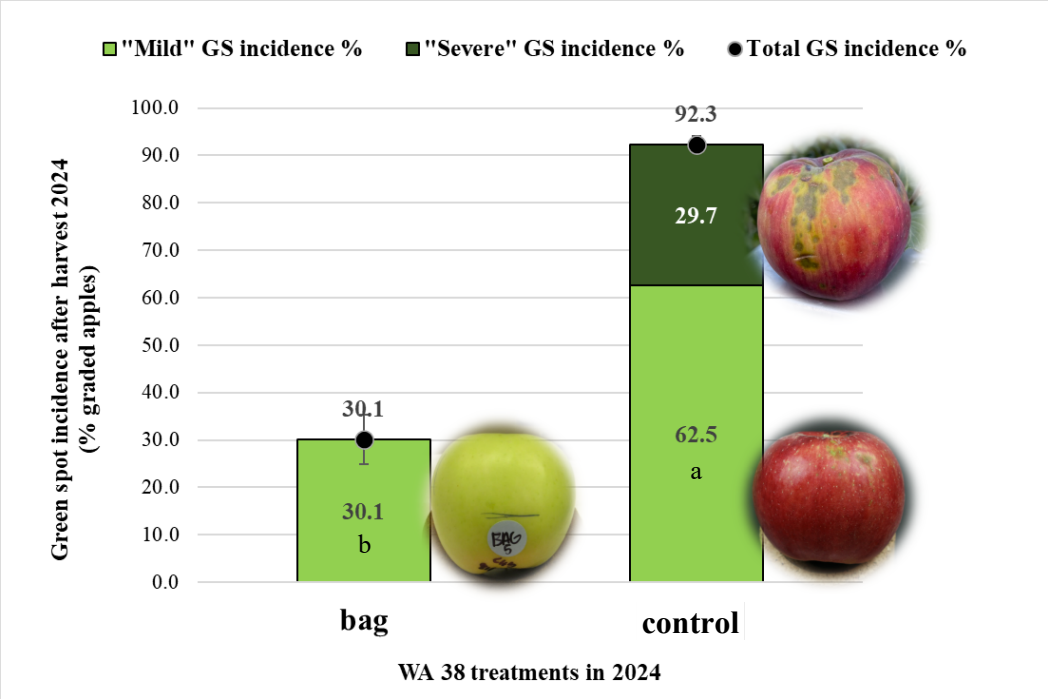

Figure 5: *'WA 38' Green spot incidence after harvest by "bag" versus "control" treatments in 2024. Light green shaded bars represent the proportion of "mild" GS, and dark green shaded bars represent the proportion of "severe" GS over the total incidence. Total incidence (%) in each treatment combination is reported above each bar and indicated by a round black marker with an associated error bar representing the standard error. Separation of means are indicated by lowercase italics letters for significance of p< 0.05. On the right side of each column bar the corresponding example of GS grading is reported. For more details consult Sheick et al., 2022.*

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EXECUTIVE SUMMARY

Project Title: Finding peel surface properties linked with green spot of 'WA 38'

***Keywords*:** green spot, bags, lenticel, acridine orange, wax, cutin.

Abstract:
Our research focused on two main objectives: understanding the role of cuticle deposition during the development of 'WA 38' apples compared with the two parents ('Enterprise' and 'Honeycrisp') and studying the development of lenticels. To study 'WA 38' green spot (GS), we utilized trees grafted on G.41 that, in previous trials carried out at Sunrise farm (SRO) showed a higher percentage of fruit affected by "Severe" GS than Nic29. This tendency has also been confirmed in 2024.
We better defined the onset of Green Spot (GS) and we were able to determine a new early symptom of Green Spot. We named it "green halo", this symptom was first observed on 6/4/2024 = 47 DAFB. Furthermore, we noticed three differences in apple growth among varieties across the 18 weeks we collected measures. Fruit growth in the green spot region (GS) is inhibited, as indicated by slight depressions of the fruit surface in those regions where green spots form. This confirms the results of the previous project. Preliminary results showed that cuticle mass per unit area is higher within green spot areas.
The results from previous studies about "bag" apples were confirmed, in particular, in 2024 the early bagged fruits did not develop signs of "Severe" green spot, while the "control" apples showed 29.7 % of "Severe" symptoms (culled apples). No significant differences were found in the incidence of GS within each bearing wood. Lenticels in "bag" fruit presented a higher Acridine Orange (AO) infiltrated area than the "control" ones at 98 and 155 DAFB.

PROJECT OUTCOMES

Manuscript:

- A manuscript explaining the difference in the cuticle and a possible theory on the development of green spot will be written as soon as possible (beginning of 2025), and it will be considered part of this report.