Increase in Epidermal Planar Cell Density Accompanies Decreased Russetting of ‘Golden Delicious’ Apples Treated with Gibberellin A₄+7

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Abstract. A 2-year study was conducted in a ‘Golden Delicious’ (Malus × domestica Borkh.) orchard with a high incidence of physiological fruit russetting to examine the effect of gibberellin A₄+7 (GA₄+7) on apple epidermal cell size. Beginning at petal fall, four sequential applications of GA₄+7 (0, 15, or 30 mg L⁻¹) were applied to whole trees every 7–10 days with an air-blast sprayer at a volume of ≈1000 L ha⁻¹. Fruit epidermal tissue samples were taken approximately monthly beginning 1 week after the fourth application. Tissue was treated in the laboratory with an enzyme mixture to remove side facing the vertical axis of the tree trunk) changes in mean thickness and number of cortical cells. At my knowledge, the effect of GA₄+7 on epidermal cells, of multiple applications of GA₄+7, has not been quantitatively examined. Thus, the objective of this work was to determine whether the decrease in russetting of ‘Golden Delicious’ apples from multiple applications of GA₄+7 was accompanied by an increase in epidermal planar cell density (number of epidermal cells per unit peel surface area).

Materials and Methods

Experiments were conducted in 2007 and 2008 in a uniform, mature, commercial orchard of ‘Golden Delicious’/‘M.M.106’ (‘MM.106’) trees growing in loamy sand near Mattawa, WA (lat. 46°39’16.58”N, long. 119°52’32.12”W; elevation 192 m). Trees were irrigated by undertree impact sprinklers and fruit from trees in most areas within this 13.2-ha block had a 70% to 80% historical incidence of russetting (≈7 of 10 years). The majority of the harvested fruit had calyx and/or shoulder russetting sufficient to cause reduction in grade). Treatment dosages of 0, 15, or 30 mg L⁻¹ GA₄+7 (ProVide®; Valent Biosciences Corp., Libertyville, IL) were applied topically to whole trees at first petal fall (FPF) and every 7–10 days thereafter for four sequential applications. Each treatment was applied to three rows of ≈20 trees per row with an orchard air-blast sprayer at a volume of ≈1000 L ha⁻¹. Treatment at the rate of 0 mg L⁻¹ was water only. Two untreated buffer rows separated each treatment row in a randomized block design.

Fruit tissue sampling. Fruitlet collection for peel tissue sampling began 1 week after the fourth (final) GA₄+7 application. In 2007, the sampling dates were 8 June, 18 June, 17 July, and 17 Aug. In 2008, the sampling dates were 12 June, 16 July, 13 Aug., and 10 Sept. Four fruit of similar size on the east side of each of four trees within each treatment row were removed and handled only by the stem and calyx end so as not to disturb the epidermal tissue. During the first two sampling dates, whole fruit were too small to be held securely in foam-pocketed trays; thus, fiber trays were modified as follows. To hold the apple securely on the tray and keep it from rolling around and disturbing the surface, a small hole 5 mm in diameter was punched through the bottom of each cup on the fiber tray. The stem of the apple was pushed through the hole and clamped on the opposite side with a small binder clip to secure it snugly against the fiber tray. In July and August, fruit were large enough to fit snugly in foam cells with the interior side of the fruit (side facing the vertical axis of the tree trunk) facing up. Fruit fitted in foam cells were placed in fiber cartons with lids and transported to the laboratory.

Fruit sampling for russet evaluations. At harvest, 3–5 d after the last tissue sample
date, 10 apples of approximately the same size were removed from 10 trees from each treated row. Fruit were transported to the laboratory and kept at 1.1 °C until further examination. Each apple was rated for russet severity (percent of fruit surface) by location (stem end or calyx end) within 10 d of harvest. 

2007: sample preparation for light microscopy. In the laboratory, the diameter of each apple was measured across the widest part of the shoulder. Two fruit per tree closest to the mean fruit diameter within each treatment replication were selected for epidermal tissue excision and processing (n = 24). From each apple, a longitudinal slice 5–8 mm wide was cut from the middle of each whole fruit roughly perpendicular to the axis of greatest sun exposure. A single epidermal section 8 mm long and 2 mm thick was excised from opposite sides of each slice at the widest diameter. Both sections were placed into a single plastic vial containing 5 mL of an enzyme solution containing pectinases [Enzyme Commission (EC) 3.2.1.15], cellulases (EC 3.2.1.4), and pectin lyases (EC 4.2.2.10) (Sigma-Aldrich, St. Louis, MO) formulated according to the method of Ju and Bramlage (1999) to remove cellular material. This enzyme solution was kept at 23 °C and changed weekly for 3 weeks. After enzymatic removal of extracuticular debris, isolated cuticles were kept at 23 °C in distilled water containing 0.01% sodium azide (NaN₃) as an aid in conferring microbial growth (Lichtstein and Soule, 1943). 

To prepare for imaging, isolated cuticles were rinsed for several minutes in clean destilled water. Each cuticle was then mounted in water on a glass slide with a glass coverslip and examined using a stereo-microscope (Model SXZ12; Olympus Corp., Tokyo, Japan) fitted with a CCD digital camera (Model CoolSNAP cf; Photometrics, Tucson, AZ). The ocular reticle (100 divisions) was calibrated with a 10-mm stage micrometer (pitch 0.1 mm) at the magnification used for image capture. After selecting a representative area, a brightfield image was recorded for further image processing. All cuticle sections were imaged using the same magnification.

Images were processed using Image-Pro Plus (Version 4.5; Media Cybernetics, Inc., Bethesda, MD). The 24-bit TIFF color images were separated into individual eight-bit RGB (red, green, blue) channels and the green channel selected for measurements. Gray-scale images were printed and quadrants drawn on each print. To minimize observer bias, the top right quadrant of each printed image was always used for counting individual cells. Two observers counted the number of individual cells within the chosen quadrant by placing a red dot on each counted whole cell and a green dot on each partial cell using a felt tip marker.

2008: sample preparation for scanning electron microscopy. Additional tissue samples were taken every 7–10 d, beginning at petal fall, using fruit from untreated trees. After the last treatment application, samples were taken every 3–4 weeks on the dates indicated previously. Samples were processed similarly to those of the previous year with several modifications. For fruit 15 mm or less in diameter, the fruit was cut equatorially into three sections in a vacuum (viscously) thickness. The middle third of the fruitlet was quartered longitudinally. Individual quarter sections were then processed as described subsequently. From fruit greater than 15 mm in diameter, a longitudinal slice 5–8 mm wide was cut from the center of each whole fruit, roughly perpendicular to the axis of greatest sun exposure. A single epidermal section 8 mm long and 2 mm thick was excised from each (opposite) side of each slice at the widest diameter. One section was set aside momentarily for further processing in preparation for examination using SEM, and the other section was placed into a single plastic vial containing 5 mL of the enzyme mixture as described previously. Vials containing apple tissue sections in enzyme solution were shaken gently using an environmental shaker kept at 38 °C. After each week in enzyme solution, samples in vials were sonicated for 10 min in a water bath kept at 38 °C before changing the solution. This was repeated until the cuticle sections were free of any discernible particulate matter in the solution after sonication. Isolated cuticles were rinsed for several minutes in clean distilled water before air drying on a clean glass slide with a glass coverslip to prevent excessive curling. Evaluation of fruit cuticles using scanning electron microscopy. Surface morphology was examined from the remaining section of peel tissue excised previously according to the method of Curry (2005) with the following modifications. From the center of each piece of peel tissue, a section of cuticle 4–5 mm in diameter and 0.2 mm thick was shaved by hand using a 0.012-mm thick double-edge stainless steel razor previously rinsed with acetone and air-dried to remove any residual oil. The shaved cuticle sections were fixed to a 24-mm aluminum stub using double-sided carbon tape by pressing the edges of the section onto the tape using a pair of fine-tipped tweezers under a stereomicroscope. The stub was placed in a glass glasper decorator containing packaged silicon gel and kept at 20 °C and 1.3 × 10⁵ Pa for 48 h or until further treatment. The time between first fruit incision and placing tissue under low vacuum in the glass desiccator was less than 2 min.

Before SEM evaluation, mounted tissue was coated with a gold/palladium alloy using a Desk II cold sputter coater (Denton Vacuum Inc., Morristown, NJ) fitted with a tilting omni-rotating head. With the sample 47 mm from the gold/palladium target, a coating thickness of 20 nm was achieved after 70 s at 40 mA and 2.6 Pa. Coated samples were kept in a vacuum desiccator and held under low vacuum at 1.3 × 10⁴ Pa and 20 °C until microscopically examined using a Tescan Vega-II Model 5136LM Scanning Electron Microscope (Tescan, s.r.o., Brno, Czech Republic) equipped with both secondary and back-scattered electron detectors. Unless otherwise noted, images were obtained at 10 kV and 7.4 × 10⁹ Pa. Similarly, isolated, air-dried cuticles from the enzymatic treatment were fixed onto the aluminum stub with the cortical side facing up. Tissue was then processed for SEM examination as previously described. Epidermal cells were counted by printing the gray-scale SEM image and tagging individual cells with colored markers as previously described. Individual cell measurements were made using the SEM software (Vega by outlining the cell “pocket” just inside the raised portion (cell imprint) of the cuticle with the drawing tool. The program calculated the perimeter and planar area of each epidermal cell imprint. At least 20 cells per image were measured from three areas of each isolated cuticle and the means and errors recorded. Analysis of variance was performed and graphs generated using Systat statistical software (Version 12.0, Systat Software, Inc., Chicago, IL). Error bars within each graph represent + or ± SEM (SE).

Results and Discussion

Fruit russetting. In 2007, fruit russetting in the section of orchard used for this study was insufficient to establish treatment differences (data not shown). Because russetting across the region was less, this effect was likely related to climate. Nevertheless, epidermal cell counts were recorded so that planar cell density could be calculated and compared. In 2008, treatment differences in russetting were apparent. Most of the russetting occurred on the distal half of the fruit (calyx end) with 77%, 31%, and 13% of the fruit having more than 20% russetted peel for GA₄+7 treatments concentrations of 0, 15, and 30 mg L⁻¹, respectively. At the highest dosage concentration of applied GA₄+7, 74% of apples had no russetting. Epidermal cell density by transillumination microscopy. 2007. A gray-scale image of the green channel (RGB) from a representative brightfield image similar to those from which planar epidermal cell density was determined for the three treatments in 2007 is shown in Figure 1. After examining all three channels, the green channel was chosen because of its slightly improved clarity (data not shown). The white dotted lines indicate the quadrant from which cells were counted. Obviously, the lines transected many cells. Therefore, transected cells were counted as half and the number of half cells was divided by two and added to the number of whole cells in the quadrant. Subsequent analysis indicated the planar cell density (number of cells per mm²) determined by this “half-cell” method was within 14% of the planar cell density determined by counting only the number of whole cells within the same approximate quadrant and then determining the exact area encompassing the cells to use as the divisor for density calculations (data not shown).

Planar cell densities for ‘Golden Delicious’ epidermal tissue 1 week after the fourth GA₄+7 treatment shown in Figure 2. Analysis of variance indicated no difference between observations. Only results from Observer B indicated
a difference among treatments; namely, the highest treatment rate resulted in the greatest epidermal planar cell density. It is likely there was insufficient definition in the images from which number of cells was counted. Bar is 10 μM.

![Graph of planar cell density vs. treatment application rate (two independent observers) from 'Golden Delicious' apple epidermal tissue sampled on 8 June 2007 and enzymatically treated to remove cell debris. Bars indicate ± SE.](image)

![Graph of planar cell area and planar cell density vs. treatment application rate from 'Golden Delicious' apple epidermal tissue sampled on 12 June 2008 and enzymatically treated to remove cell debris. Using this method, a graph of planar cell area and cell density was constructed for cuticles sampled on 12 June 2008 and enzymatically treated with GA4+7 (Fig. 4). Differences among treatments are clear. Epidermal planar cell density increased by 14% and 27%, whereas epidermal planar cell area decreased by 18% and 31% for application rates of 15 mg L⁻¹ and 30 mg L⁻¹, respectively. Not unexpectedly, there was greater variability in both planar cell area and planar cell density for the GA4+7 application rate of 15 mg L⁻¹ than for both higher and lower rates. Possibly, this is related to activation threshold. As the apple enlarges, 1) cuticle thickens and may reduce penetration of aqueous a.i.; and 2) number of epidermal cells beneath each microdroplet decreases, thereby reducing number of affected cells. This is made clearer by the data in Figure 5, which shows cutin imprints of epidermal cells during the first 30 d of fruitlet development. The area within the 100-μm diameter circle (e.g., microdroplet) encompasses fewer, albeit larger, epidermal cells as fruitlet diameter increases. Thus, less a.i. may contact fewer cells. Whether a threshold exists, however, or whether a threshold changes with development is not known. Other factors that may influence efficacy during the first few weeks of fruit development are ambient conditions that alter drying time, the presence of other topically applied compounds, and increased leaf area (reduced spray penetration). In this particular orchard, canopy development at the FPF stage was quite different from 3 weeks later (E. Curry, personal observation).

![Graph of planar cell surface area vs. fruitlet diameter of untreated 'Golden Delicious' apple cuticles isolated during the first ≈5 weeks of fruitlet development.](image)
Fig. 6. Scanning electron micrographs of the interior surface (flesh side) of representative sections of untreated ‘Golden Delicious’ apple fruit cuticle sampled on 12 June (A), 16 July (B), 13 Aug. (C), and 10 Sept. (D) and enzymatically treated to remove cell debris. Note thickening and multilayered cutin on successive sample dates. Bar is 20 μm.
With smaller epidermal cells, one would also expect microcracks to be thinner, shorter in length, and perhaps less invasive as suggested by the images in Figure 7C–D assuming that microcracking is mainly a function of epidermal cell division and/or sub-epidermal cell enlargement.

Generally, environmentally based russet development is either a direct response of the fruit epidermal cell to desiccation or an indirect response to excessive moisture. Response to desiccation may occur by: 1) physical damage resulting in exposure of cells to air with less than 100% humidity; 2) injury of epidermal cells resulting in arrested cuticle development; or 3) disruption of the protective wax layer resulting in a less than optimized water vapor barrier for given conditions. Excessive moisture, in contrast, may elicit russetting by one of two mechanisms. Standing water may increase barrier for given conditions. Excessive moisture smothering of localized, high-humidity microenvironments on cuticle thickness and composition as well as sub-epidermal cell organization may provide insight into postharvest disorder development and quality loss in storage.

Conclusions

Data from this 2-year study using fruit from an historically high-russetting ‘Golden Delicious’ orchard suggest GA4+7 reduces environmental peel russetting by reducing epidermal cell size. Transillumination microscopy did not permit accurate measurement of ‘Golden Delicious’ fruit epidermal cell planar area beyond 6–8 weeks after anthesis because isolated cuticles became thicker and multilayered causing chromatic aberrations. On the other hand, evaluating epidermal cell planar measurements using SEM was clear and more accurate. Further work to examine the effect of localized, high-humidity microenvironments on cuticle thickness and composition as well as sub-epidermal cell organization may provide insight into postharvest disorder development and quality loss in storage.

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