PROGRESS REPORT FOR 2000-01

PROJECT NO.: 8937

TITLE: Antioxidant Defense Systems for Protection Against Sunburn in Apple

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SIGNIFICANT FINDINGS:

- There appear to be differences in the antioxidant contents of fruit peel from different apple cultivars.
- Ascorbic acid field sprays did not appreciably reduce sunburn incidence on Fuji apples in 2000.
- The contribution of ascorbic acid to the total antioxidant activity of apple fruit peel appears to be significant, but variable, depending on developmental and environmental factors.
- Compared to several other commercial surfactants, SylGard 309® appears to be the most effective surfactant for uptake of topically applied ascorbic acid into apple fruit.
- Exposure to varying light and temperature regimes under controlled laboratory conditions suggests that sunburn-like symptoms and oxidative damage only occur when apple fruit tissue is exposed to both high light and high temperatures.

OBJECTIVES:

- Screen selected apple genotypes for antioxidant levels during fruit development to identify genotypes to be used as breeding material for stress resistance in the WSU apple-breeding program.
- Determine the efficacy of topically applied antioxidants in reducing photooxidative damage to fruit of selected apple varieties.
- Identify limiting steps in the pathways of ascorbic acid biosynthesis and recycling in fruit.

PROCEDURES:

Field plots and sampling

In June 2000, we established research plots in double-row (N-S) V-trellis, Fuji (Naga-fu 12)/M.9, 5th-leaf Granny Smith/M.9, and 2nd-leaf Pink Lady/M.9 blocks in a commercial orchard near Vantage and in a single-row (SW-NE) hedgerow, 3rd-leaf Fuji (BC2)/M.9 commercial orchard near Othello. In the Vantage plots we collected fruit peel samples from: 1) the sunlight exposed side of exposed fruit, 2) the shaded side of these same exposed fruit, 3) the sunlight exposed side of fruit under evaporative cooling for sunburn protection, and 4) the sunlight exposed side of mostly shaded fruit. For each of these varieties, samples were collected at bi-weekly intervals from mid-July until commercial harvest. We also collected sunburn damaged peel from fruit that were sunburned to different degrees and for

different durations. Similar sample collections were performed at Othello, except that very little EC was used at this orchard. Leaf samples were also collected at both Vantage and Othello, in order to relate what we found biochemically in fruit peel with that in leaf tissue. We also collected fruit peel and leaf samples from several varieties and genotypes growing at the NE-193 cultivar trial at TFREC-Wenatchee.

Antioxidant sprays and sunburn

At the Vantage orchard, we applied sprays of 3% (w/v) ascorbic acid (AsA) with 0.03% (4 fl oz/100 gal) SylGard 309® as surfactant, to Fuji trees with a hand wand sprayer at 50-100 GPA, either as: 1) weekly full-season sprays (July 18-Sept 29), 2) weekly late-season sprays (Aug 25-Sept 29), or 3) intermittent sprays only when high temperatures were forecast (weekly from July 18-Aug 31). A control spray of SylGard was also applied. These treatments were replicated in a 4-block RCB design. No fruit peel samples were taken from these treatments until harvest. At harvest, counts were made of sunburned fruit of both E- and W-facing fruits greater than 5 feet above the ground. Each sunburned fruit was categorized by sunburn severity from no sunburn (SB0) to severe browning (SB3). Sprays of AsA were also applied at the Othello orchard to evaluate the short-term biochemical changes in fruit peel of treated fruit.

Sample analysis

All fruit samples were immediately peeled in the field directly into liquid nitrogen to avoid post-sampling oxidation. Frozen peel and leaf samples were transported on dry ice and stored in a –80C freezer until analyzed. Peel and leaf samples are in the process of being analyzed for aqueous- and methanol-extracted total antioxidant activity (Cano et al., 1998), oxidized and reduced forms of AsA (Foyer et al., 1983) and glutathione (Griffith, 1980), ascorbate-glutathione cycle enzyme activities (Foyer and Halliwell, 1976; McCord and Fridovich, 1969; Miyake and Asada, 1992; Nakano and Asada, 1987), and pigments, i.e. chlorophyll (Arnon, 1949), carotenoids (Davies, 1976), and anthocycanins (Ju and Bramlage, 1999). Sunburn damaged and undamaged peel tissue also will be analyzed for protein (Levine et al., 1990; Sun and Leopold, 1995) and lipid oxidation (Du and Bramlage, 1992).

Environmental monitoring

Ambient air temperature, incoming sunlight, and surface temperatures and incident sunlight on the SW-facing side of 48 fruits exposed to full sunlight in the non-EC section of the Fuji blocks at Vantage and Othello were continuously monitored and recorded on a datalogger throughout the 2000 season. Each fruit was evaluated weekly for incidence and severity of sunburn.

Antioxidant uptake into apple fruit skin

Radiolabeled ^{14}C-AsA (1% w/v) in a range of potential surfactants was applied to fruit discs of Fuji apples under controlled conditions in the laboratory, similar to procedures described by Knoche and Bukovac (1992). A total of 2 microliters in 10, 0.25 □L, droplets, were applied to each disc. Five discs for each surfactant treatment in petri dishes were covered with a clear lid and exposed for 4 hrs under high pressure sodium lamps resulting in exposure to approximately 70% full sunlight and 108°F (42°C) surface temperature. After exposure was completed, the skin surface of each disc was washed with distilled water and the cuticle was separated from the skin by cellulose acetate peeling. Both the surface and cuticle fractions were placed in separate vials containing liquid scintillation cocktail. Then the peel was removed from the flesh of each disc and oxidized in a biological oxidizer, with the $^{14}CO_2$ trapped in liquid scintillation cocktail. The counts per minute (CPM) of radiation of all samples and fractions were determined in a liquid scintillation counter.

Controlled environment experiments
Whole fruit and/or fruit discs from Granny Smith and Fuji apples collected from the Vantage orchard were subjected to different light/temperature regimes under controlled conditions in the laboratory. After exposure, the whole fruit and/or discs were evaluated for injury and the peel was sampled for biochemical analyses as previously described.

RESULTS AND DISCUSSION:

Controlling sunburn with AsA sprays
There was little difference in the percentage of sunburn between control and AsA-sprayed trees in 2000, with 74-79% of the fruit exhibiting no sunburn in all treatments. There was 2-3% greater incidence of mild sunburn (SB1) in the untreated plots than in those that received weekly full-season AsA sprays (July 18-Sept 29), and 2-4% greater incidence of moderate sunburn (SB2) in the untreated and late-season AsA-sprayed plots than in the full-season AsA-sprayed plots. Nevertheless, these differences are minor. Nevertheless, in previous years better control of sunburn was achieved with AsA sprays.

Total antioxidant activity
A preliminary comparison was made of the contribution of AsA to the soluble (aqueous extracted) and insoluble (methanol extracted) total antioxidant activity (TAA) in the peel of Fuji apples from 1999. Most of the TAA in peel from sunlight-exposed and shaded sides of non-EC fruit and from sunlight-exposed side of EC fruit was in the soluble fraction, and not the insoluble fraction, on both Aug. 19 and Sept. 9, 1999. At the time in fruit development when AsA content was high (i.e., just prior to red color break) most of the TAA in the peel of sunlight exposed skin, whether the fruit were under EC or not, was largely contributed by AsA. In the peel from the shaded side of these fruit, or in exposed peel of fruit later in development when AsA contents were lower (i.e., during red color development), less than half of TAA appeared to be contributed by AsA. Therefore, soluble antioxidants generally, and AsA specifically, appear to be the dominant antioxidants in apple fruit peel, except during fruit development when contribution to TAA by AsA is low and susceptibility to sunburn is high. Fruit peel samples collected in 2000 will be analyzed for TAA to determine the contribution of AsA and glutathione to the antioxidant activity of different varieties at various stages of fruit development.

Ratios of TAA in aqueous and methanol fractions, compared with AsA

	----------Aug. 19, 1999----------			----------Sept. 9, 1999----------		
	Exposed	Shaded	EC, Exposed	Exposed	Shaded	EC, Exposed
$TAA_{aq}/TAA_{aq+meth}$	0.64	0.73	0.65	0.73	0.77	0.60
$TAA_{meth}/TAA_{aq+meth}$	0.36	0.27	0.35	0.27	0.23	0.40
$AsA_{red}/TAA_{aq+meth}$	0.61	0.36	0.57	0.36	0.22	0.41
AsA_{red}/TAA_{aq}	0.96	0.49	0.87	0.50	0.28	0.68

Cultivar screening
Initial measurements were made of ascorbic acid (AsA), glutathione, and total antioxidant activity (TAA) of fruit peel from different genotypes in the NE-193 cultivar trial at TFREC-Wenatchee from samples collected on July 28 and Aug. 18, 2000. The reduced plus oxidized AsA (AsA_{red+ox}) content was highest in Honey Crisp (only on July 18, because there were no fruit available on the trees in Aug.) and a selection from Kazakhstan (only on July 28). Generally, Fuji and Gala had the lowest

AsA contents on both sampling dates. Only 11-38% of the total AsA was in the free-radical scavenging reduced form in these cultivars. There was little difference in total glutathione (reduced GSH + oxidized GSSG) content among the cultivars, but glutathione content is only 1/500th-1/1000th that of the AsA content in the fruit peel of these cultivars. For all cultivars, most (73-88%) of the TAA ($TAA_{aq+meth}$) was in the aqueous fraction (TAA_{aq}), with only a minor contribution to TAA in the lipid-soluble methanol fraction (TAA_{meth}). There were differences in the contribution by AsA to the TAA of peel from these cultivars, with Honey Crisp having the highest contribution to total TAA coming from AsA ($AsA_{red}/TAA_{aq+meth}$ = 61% and AsA_{red}/TAA_{aq} = 74%) on July 28, whereas on Aug. 18, Cameo and Braeburn had most of their TAA contributed by AsA. Fuji and Gala usually had the lowest contribution to TAA coming from AsA. These results may reflect differences in the free-radical scavenging ability among these cultivars. Fruit peel and leaf samples from the NE-193 trial were collected on one later date and are in the process of being analyzed.

Total AsA, glutathione, and TAA in fruit peel of selected apple cultivars.

	GD	Cameo	Brae	Gala	Fuji	HC	Kazak
	July 28, 2000 samples						
AsA_{red+ox} (□mol/g FW)	19	26	18	11	11	44	62
AsA_{red}/ AsA_{red+ox}	0.26	0.22	0.28	0.26	0.20	0.27	0.11
GSH_{red+ox} (nmol/g FW)	209	235	203	223	218	235	238
$TAA_{aq+meth}$ (□mol/g FW)	16	14	10	13	15	20	21
TAA_{aq}/ $TAA_{aq+meth}$	0.76	0.73	0.74	0.74	0.76	0.82	0.85
TAA_{meth}/ $TAA_{aq+meth}$	0.24	0.27	0.26	0.26	0.24	0.18	0.15
AsA_{red}/ $TAA_{aq+meth}$	0.31	0.40	0.47	0.22	0.15	0.61	0.33
AsA_{red}/ TAA_{aq}	0.41	0.55	0.64	0.30	0.19	0.74	0.39
	August 18, 2000 samples						
AsA_{red+ox} (□mol/g FW)	16	21	23	9	12	–	10
AsA_{red}/ AsA_{red+ox}	0.32	0.33	0.27	0.37	0.30	–	0.38
GSH_{red+ox} (nmol/g FW)	156	166	149	158	49	–	195
$TAA_{aq+meth}$ (□mol/g FW)	9	9	7	10	12	–	13
TAA_{aq}/ $TAA_{aq+meth}$	0.82	0.88	0.86	0.85	0.82	–	0.87
TAA_{meth}/ $TAA_{aq+meth}$	0.18	0.12	0.14	0.15	0.18	–	0.13
AsA_{red}/ $TAA_{aq+meth}$	0.59	0.83	0.78	0.35	0.30	–	0.28
AsA_{red}/ TAA_{aq}	0.72	0.95	0.91	0.41	0.37	–	0.33

GD=Golden Delicious, Brae=Braeburn, HC=Honey Crisp, Kazak=selection from Kazakhstan

Antioxidant uptake

In a preliminary experiment, uptake of radiolabeled ^{14}C-AsA into the peel was most effective with SylGard 309 (SG), which also left the least amount of ^{14}C-AsA on the surface of the skin or within the cuticle. Other surfactants included Latron AG-98 (AG), Latron B-1956 (B), Fulvic Acid (FA), Regulaid (R), SylTac (ST), TriFol (TF), and X-77 (X). However, to optimize the efficacy of SylGard-assisted uptake of AsA (or of any other a.i.), we will test SylGard at different concentrations and under varying environmental conditions.

Uptake of AsA in different surfactants into apple fruit discs.

	% difference from ^{14}C-AsA in water (without surfactant)							
Fraction	AG	B	FA	REG	**SG**	ST	TF	X
Surface	202	127	314	232	**-49**	163	447	104
Cuticle	207	158	152	167	**22**	231	200	83
Peel	430	397	64	290	**1488**	451	106	400

Controlled environment experiments

When whole Granny Smith apples were exposed to controlled environmental conditions in the laboratory, sunburn symptoms only appeared (after 3.5 hrs) when fruit were treated both with high light (70% full sunlight with 1000W high pressure sodium lamp) and skin temperatures of 118-122°F (48-50°C). Treatment with high light at lower skin temperatures (99-102°F or 37-39°C), or with no light and high skin temperatures (124-127°F or 51-53°C) did not produce sunburn symptoms after 6 hrs. Protein oxidative damage in peel from the exposed side of the fruit, as measured by carbonyl content, only increased in the sunburned fruit that were exposed to high light and heat. Treatment with UV-A lamps at low air temperature neither promoted sunburn symptoms nor increased carbonyl content. These results suggest that both high light and high temperatures are required to induce sunburn and oxidative damage. This confirms our hypothesis that sunburn is the result of photooxidative injury of heated skin tissue. Nevertheless, we will repeat this experiment on Granny Smith and other varieties.

Protein oxidation in apple fruit exposed to different light and temperature conditions.

	Control	HL/HT	HL/LT	HT	UV-A
Carbonyl content (nmol/mg protein)	15.5	24.7	7.5	7.5	6.3

HL/HT=high light/high temp, HL/LT=high light/low temp, HT=high temperature only, UV-A=ultraviolet A light

Environmental monitoring

Although analysis of field environmental data is incomplete, early evaluation of the contribution to sunburn by incident light intensity on fruit skin and temperature of fruit skin indicates that light is the more dominant factor.

Note: In all of the areas of study reported above, sample analyses and evaluation of environmental data are still in progress, and will be subsequently reported.

CONCLUSIONS:

- Different apple cultivars may be protected against oxidative damage to the fruit because they contain different levels of specific antioxidant compounds.
- Ascorbic acid sprays may only be effective at significantly reducing sunburn of apple fruit when applied at specific, but still undetermined, environmental conditions.

- Optimization of ascorbic acid uptake, or of any other topically applied compound, by apple fruit skin greatly depends on surfactant efficacy.
- Sunburn of apple fruit is the result of photooxidative stress imposed by high light and temperature, resulting in oxidation of vital biochemical compounds within the fruit skin.

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