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Determining the Potential To Breed for Enhanced Antioxidant Status in *Malus*: Mean Inter- and Intravarietal Fruit Vitamin C and Glutathione Contents at Harvest and Their Evolution during Storage

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Vitamin C (L-ascorbate, L-ascorbic acid; L-AA) and glutathione (GSH) are major hydrophilic antioxidants in plants with important roles in stress resistance and nutrition. To evaluate the potential for breeding for enhanced levels of these compounds, a comprehensive screen of the fruit from some 31 apple (*Malus*) cultivars has been carried out to determine the biodiversity present in the mean inter- and intracultivar concentrations of both the oxidized and reduced forms of these compounds, as well as the impact of storage on their concentrations. It is noted that despite limited variation at harvest, cultivars differed substantially in their ability to maintain L-AA levels during storage, primarily due to the loss of L-AA by "low-vitamin C" cultivars. Generally, cultivars that could maintain their L-AA and GSH pools also had better storage properties. Interestingly, there was also a correlation between fruit vitamin C contents and the harvest date, such that cultivars with the highest vitamin C contents were harvested latest in the season and the lowest contents were found among the early varieties. Correlations with other physiological parameters, however, were too weak to serve as useful predictive tools.

KEYWORDS: ***Malus*; fruit; antioxidant; biodiversity; harvest; shelf life; cold storage; vitamin C; glutathione**

INTRODUCTION

The Laboratory of Fruit Breeding and Biotechnology, KU Leuven, maintains an extensive apple (*Malus* × *domestica* Borkh.) germplasm collection on its 23 ha field station. One of its research aims is to improve fruit (nutritional) quality by enhancing antioxidant levels. During the processes of adaptation and acclimation to a wide variety of environmental stresses (high light, pollutants, drought, cold, etc.), there is a general up-regulation of the plant's antioxidant defense systems (*1*−*7*). Therefore, it is widely expected that crop varieties with increased antioxidant contents will have better fruit qualities, yield, and storage characteristics as a consequence of an improved performance under suboptimal environmental conditions. In addition, there may be benefits to the consumer because the increased dietary consumption of a range of antioxidant compounds is associated with a decreased incidence of a number of long-term chronic illnesses (*8*−*10*).

In this study we were particularly interested in looking at fruit vitamin C (L-ascorbate, L-AA) and the tripeptide glutathione (γ-glutamyl cysteinyl glycine, GSH) concentrations. L-AA is ubiquitous in living plant cells, and together with GSH forms an integral part of the (photo)oxidative stress defense system. Apart from direct antioxidant functions, however, both L-AA and GSH are also associated with the regulation of a wide range of metabolic functions including plant growth and development, hormone function, enzymatic activities, and transcription (for reviews see refs *11*−*16*). The health-promoting benefits of L-AA are also well documented and range from reduced incidence of the common cold to the prevention of scurvy to reduced incidence of certain cancers (reviewed in ref *17*).

Apart from a recent publication from our own group, however (*18*), little is known about L-AA and GSH homeostasis in *Malus* tissues. Although fragmentary data are available for the L-AA contents of apple fruits (summarized in ref *11*), these results have often been obtained using differing and outdated methodologies and using material grown under different conditions or in different seasons. We have been unable to find any information describing the GSH contents of fruits. Here we report the screening of 31 commercial varieties of apple (*Malus* × *domestica* Borkh.), grown in a single season at a single location in Belgium and using methodologies optimized for this analysis (*19*). We have further quantified not only reduced L-AA and GSH levels but also the concentration of the oxidized forms of these antioxidants, that is, dehydroascorbate (DHA) and glutathione disulfide (GSSG), respectively. DHA and GSSG are

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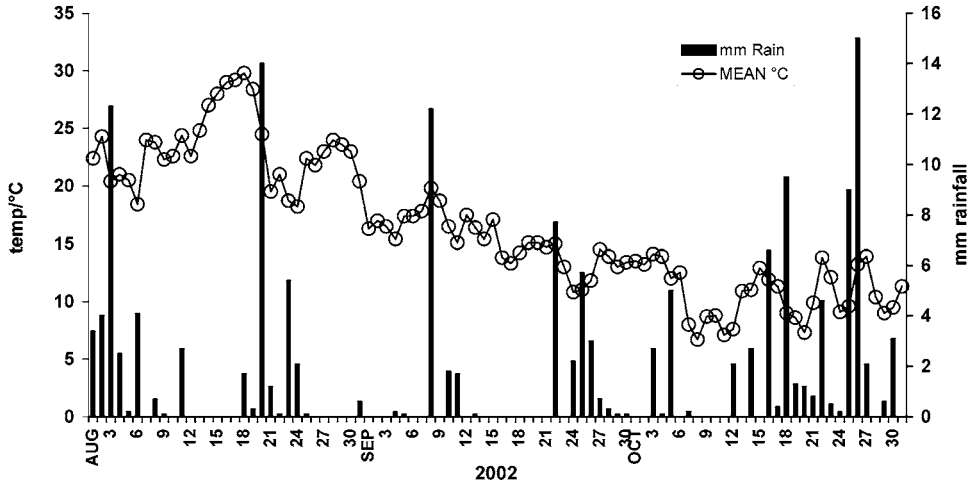

Figure 1. Mean temperature and rainfall for field station where apples were grown (Aarschot, Belgium), for the season August–November 2002.

Table 1. Overall Mean Values, Concentration Ranges, and Fold Variation for (**A**) Fruit Fresh Weights, (**B**) Fruit L-AA, and DHA Contents, and (**C**) GSH and GSSG Contents for the 31 *Malus* Cultivars Analyzed, at Harvest, after 10 Days of Storage at Room Temperature (Shelf Life, SL), and after 3 Months of Storage at 1 °C (Cold Storage, CS)[a]

(A) Fruit Fresh Weights

		weight	
sampling time		gfw	SD
harvest	mean	168.0^{A}	±69.0
	range	85.0–288.9	
	fold variation	3.4	
shelf life	mean	165.2^{A}	±58.2
	range	77.9–308.7	
	fold variation	4	
cold-storage	mean	164.6^{A}	±59.5
	range	76.8–312.2	
	fold variation	4.1	

(B) Fruit L-AA and DHA Contents

		L-AA		DHA		total L-AA		
sampling time		nmol/gfw	SD	nmol/gfw	SD	nmol/gfw	SD	% DHA
harvest	mean	626.0^{A}	±267.3	99.3^{A}	±145.6	722.1^{A}	±303.5	12.1^{A}
	range	296.5–1283.1		0–265.7		400.9–1448.2		0–25.1
	fold variation	4.3		–		3.6		–
shelf life	mean	423.9^{B}	±333.9	46.2^{AB}	±35.8	468.5^{B}	±349.6	11.9^{A}
	range	83.7–1227.2		0–128.5		108.5–1314.4		0–34.5
	fold variation	14.7		–		12.1		–
cold-storage	mean	547.7^{AB}	±378.2	40.2^{B}	±22.6	586.5^{AB}	±390.1	8.2^{A}
	range	134.2–1514.6		2.9–86.3		157.2–1589.1		0.3–12.0
	fold variation	11.3		29.8		10.1		40.0

(C) GSH and GSSG Contents

		GSH		GSSG		total GSH		
sampling time		nmol/gfw	SD	nmol/gfw	SD	nmol/gfw		% GSSG
harvest	mean	51.8^{A}	±22.3	9.1^{A}	±12.0	59.3^{A}	±27.0	12.9^{A}
	range	20.7–90.9		0–38.4		27.8–129.2		0–46.0
	fold variation	4.4		–		4.6		–
shelf life	mean	33.5^{B}	±16.6	8.1^{A}	±8.3	41.3^{B}	±18.8	18.0^{A}
	range	12.2–77.7		0–26.7		14.3–85.7		0–53.9
	fold variation	6.4		–		6.0		–
cold storage	mean	87.9^{C}	±30.4	25.7^{B}	±23.5	112.8^{C}	±50.0	19.6^{A}
	range	47.9–128.4		3.8–84.5		64.4–244.5		2.3–42.3
	fold variation	2.7		22.2		3.8		18.4

[a] Values followed by the same letter are not significantly different from each other ($P < 0.05$). Values represent the overall mean values from the analysis of 10 individual fruit, randomly chosen of each variety, and analyzed either on the day of harvest, after 10 days at room temperature (shelf life), or after 3 months at 1 °C in a cool room. The 31 different varieties examined are the same as specified in **Table 2**. Statistical correlations were calculated using the Student's *t* test.

considered to be markers for the degree of oxidative stress experienced by the cell. Finally, we determined the impact of storage conditions on the concentrations of the L-AA/DHA and GSH/GSSG redox pairs and the relationships between L-AA and GSH in different tissues. These results are discussed in the context of helping to focus and develop future breeding strategies.

Table 2. Mean Values for L-AA, DHA, GSH, GSSG, Soluble Sugar (as %Brix), Hardness, pH, and Acidity for Commercial Belgian Apple Varieties in 2002

variety	harvest 2004	gfw	SD	L-AA	SD	DHA	SD	GSH	SD	GSSG	SD	Brix	SD	hardness	SD	pH	SD	acidity (mL of NaOH)	SD
Sunrise	Aug 9	124.4	±11.7	664.4	±96.4	11.4	±16.4	27.0	±3.6	0.9	±1.6	11.7	±0.4	3.3	±0.1	3.5	±0.1	22.0	±2.2
Gravenstein	Aug 9	91.4	±22.5	1056.1	±195.3	91.4	±61.6	70.4	±21.2	0.0	±10.8	13.1	±0.9	4.6	±0.7	3.3	±0.1	30.6	±7.1
Retina	Aug 14	190.7	±42.1	453.2	±89.7	55.5	±18.1	73.6	±19.3	1.4	±4.1	12.8	±1.0	4.3	±0.5	3.5	±0.1	28.2	±7.1
Prima	Aug 20	113.2	±14.5	363.1	±40.9	80.7	±39.5	38.0	±7.2	0.9	±1.0	10.7	±0.6	4.2	±0.3	3.3	±0.0	28.1	±2.5
Delbare Estival	Aug 20	168.2	±16.2	462.6	±83.5	16.2	±21.6	42.7	±5.4	10.9	±4.3	10.4	±0.4	4.7	±0.3	3.8	±0.1	16.4	±1.2
James Grieve	Aug 20	231.5	±37.8	534.9	±58.8	27.4	±13.7	75.1	±8.4	10.2	±3.5	11.6	±0.9	3.6	±0.4	3.3	±0.1	40.0	±3.9
Alkmene	Aug 20	154.3	±31.9	440.9	±53.6	16.5	±9.5	5.5	±5.9	1.9	±9.9	11.9	±0.7	3.6	±0.3	3.5	±0.1	26.1	±2.0
Arlet	Aug 28	143.5	±20.8	693.1	±145.5	19.9	±167.7	72.8	±16.5	5.2	±11.2	11.1	±0.6	4.4	±0.4	3.4	±0.0	24.7	±2.4
Gala	Aug 28	136.3	±19.1	415.4	±137.5	22.7	±167.7	27.2	±5.9	4.9	±9.9	11.0	±0.8	5.0	±0.4	3.9	±0.0	12.8	±1.0
Elstar	Sept 4	173.0	±29.6	466.8	±93.6	78.8	±44.6	41.8	±4.8	5.8	±3.7	11.8	±0.7	2.9	±0.2	3.4	±0.0	29.8	±2.2
Liberty	Sept 4	137.9	±25.8	677.0	±137.5	51.8	±167.7	37.7	±5.9	6.2	±9.9	11.4	±0.5	5.3	±0.4	3.2	±0.0	34.4	±3.0
Merlijn	Sept 6	145.7	±31.0	442.3	±83.5	0.0	±37.6	31.6	±11.2	0.1	±2.8	12.4	±2.0	3.4	±0.5	3.5	±0.1	17.5	±3.9
Priscilla	Sept 6	85.0	±9.9	296.5	±54.0	104.4	±64.9	27.4	±6.5	7.5	±9.2	10.5	±0.3	5.1	±0.4	3.7	±0.0	9.0	±1.8
HL248	Sept 9	187.3	±31.3	500.1	±87.5	31.9	±97.3	52.8	±12.0	0.0	±6.5	10.9	±0.5	4.0	±0.3	3.4	±0.1	20.1	±2.7
Angold	Sept 16	261.4	±46.8	770.3	±104.5	87.3	±26.9	20.7	±5.1	0.5	±4.9	12.1	±0.8	3.6	±0.7	3.2	±0.0	32.0	±6.2
Golden	Sept 19	165.1	±25.3	937.0	±246.4	52.5	±186.3	64.2	±16.6	0.8	±13.9	11.3	±0.8	3.6	±0.2	3.5	±0.1	19.3	±1.7
Kanzi	Sept 23	205.4	±63.6	761.9	±181.1	127.4	±106.0	63.3	±9.3	2.3	±10.8	13.0	±0.8	4.1	±0.4	3.3	±0.1	28.8	±1.6
Cox	Sept 24	181.7	±33.8	811.6	±229.6	319.9	±206.4	85.6	±29.9	2.0	±31.8	11.4	±2.2	4.4	±0.7	3.5	±0.1	28.2	±3.9
Delbare Jubile	Sept 24	281.9	±37.5	804.7	±161.7	21.8	±153.9	62.1	±10.9	0.0	±4.9	12.5	±1.6	3.3	±0.3	3.5	±0.0	22.2	±2.7
Micromalus	Sept 24	3.7	±0.8	307.1	±201.7	782.9	±321.7	42.8	±4.9	5.6	±5.7	16.6	nd	nd	nd	3.3	nd	75.6	nd
Goldrush	Oct 1	131.4	±20.0	782.6	±69.0	32.4	±22.8	50.0	±8.5	13.6	±4.3	12.8	±1.0	4.9	±0.7	3.5	±0.1	29.1	±4.1
Florina	Oct 1	138.7	±49.4	561.5	±56.1	89.6	±11.2	78.7	±6.6	2.7	±1.0	13.4	±0.5	3.4	±0.3	3.5	±0.0	25.9	±1.6
Idared	Oct 1	255.6	±62.9	767.0	±49.5	0.0	±43.1	31.7	±6.6	33.6	±18.1	10.3	±0.9	3.5	±0.2	3.4	±0.0	25.4	±4.9
Rome Beauty	Oct 1	288.9	±58.7	492.4	±94.8	63.5	±18.8	45.5	±4.1	3.6	±2.8	10.1	±3.6	4.2	±1.5	3.5	±0.1	20.0	±1.7
Gloster	Oct 1	184.0	±28.7	508.0	±221.4	56.5	±58.4	71.6	±33.4	25.6	±8.0	10.4	±0.6	4.1	±0.3	3.5	±0.0	20.9	±1.1
Greenstar	Oct 2	176.3	±21.3	916.7	±143.7	71.6	±36.6	39.8	±6.7	20.6	±4.3	10.2	±0.4	3.8	±0.3	3.5	±0.0	14.7	±1.4
Jonagold	Oct 7	275.6	±28.9	642.3	±151.6	155.7	±145.2	46.7	±12.6	26.0	±18.5	12.8	±0.6	4.7	±4.8	3.6	±0.1	17.5	±2.2
Fuji	Oct 15	176.3	±36.5	470.9	±73.9	157.1	±77.5	50.6	±1.9	43.1	±7.8	13.4	±1.2	3.8	±0.3	3.9	±0.1	14.1	±2.1
Ontario	Oct 15	248.8	±45.1	1182.4	±189.4	265.7	±169.0	90.9	±14.5	38.4	±15.9	11.7	±1.0	3.3	±0.2	3.2	±0.1	37.8	±3.2
Braeburn	Oct 22	182.0	±23.7	1283.0	±31.7	95.2	±55.2	87.7	±4.5	3.5	±2.2	11.7	±0.7	4.5	±0.2	3.5	±0.1	24.8	±2.7
Pink Lady	Oct 23	132.6	±44.7	561.5	±56.1	89.6	±11.2	78.7	±6.6	2.7	±1.0	12.3	±0.4	4.5	±0.5	3.3	±0.1	25.5	±1.9

MATERIALS AND METHODS

Growth. All apples were harvested in 2002 from trees grown at a commercial field station in Aarschot, Belgium (52° N). Trees were cultivated and managed according to standard Belgian agricultural practices, involving an integrated pest management scheme.

Sampling. For the screening of fruit metabolite contents, a minimum of 30 apples per variety was harvested, obtained from between 3 and 10 trees per variety. Ten of these fruits were analyzed within a few hours of harvest, whereas the remaining apples were either kept for 3 months in standard cold-storage conditions at 1 °C ("cold storage", CS) or analyzed after storage at room temperature for 10 days ("shelf life", SL). For each individual fruit, the following measurements were taken: weight, diameter, soluble sugars content (% Brix), hardness, and acidity. The whole fruit was then simultaneously sliced radially and decored, using a commercially available slicer. Half of the slices were utilized for acidity measurements, whereas the remainder were extracted for HPLC analysis of oxidized and reduced L-AA and GSH. To accommodate for effects related to the position of the slice (and the fruit's exposure to sunlight while on the tree), slices were selected alternately around the circumference.

Metabolite Measurements. Acid-soluble metabolites were extracted by blending half of the fruit slices in 2 volumes (mL/gfw) of chilled extraction solvent (6% metaphosphoric acid/2 mM EDTA/2% PVPP). L-AA and GSH were simultaneously quantified from the selected slices by analyzing aliquots of the prepared extracts by gradient, ion suppression RP-HPLC as previously described (*19*). The oxidized forms of these two antioxidants (DHA and GSSG) were determined by the "subtractive" method after measurement of the total ascorbate (L-AA + DHA) and total glutathione (GSH + GSSG) contents following reduction with DTT (*19*).

The "hardness" of individual fruits was determined at two points on the circumference of the fruit (sunny or red and shaded or green), using a penetrometer (Bishop, fruit pressure tester, model FT327, 8 mm diameter probe). Soluble sugar content (% Brix) was determined using a portable digital refractometer, model PR-32 (Atago Co. Ltd.), with juice obtained from the same two points of the apple. Acidity measurements were carried out by juicing the remaining half of the apple slices in a commercial juicer, diluting 25 mL of the apple juice with an equal volume of distilled water, and carrying out automatic pH and acid titration measurements using a Metrohm 719 Titrina automatic pH titrator.

Meteorological Measurements. The field station where experiments were carried out also serves as a meteorological station, providing data for national weather statistics in Belgium. Mean temperatures and rainfall for the season 2002−2003 are shown in **Figure 1**. These meteorological statistics indicate that the 2002−2003 season was "normal".

RESULTS AND DISCUSSION

L-AA and GSH Analysis. In the past many of the procedures used to measure L-AA contents were inaccurate, primarily due to interference from other cellular metabolites (for discussions, see refs *11* and *20*). We have addressed these problems and optimized methodologies for the rapid gradient HPLC analysis of both L-AA and GSH in different tissues of *Malus* (*19*). This protocol represents an approximately 5-fold reduction in analysis time compared with conventional gradient HPLC analyses and allowed us to carry out a widespread screening program for these metabolites.

Sampling. Fruit L-AA and GSH levels vary according to the type of tissue being analyzed, the incident light intensity, and the canopy position (*18*, *21*, *22*). Therefore, it is necessary to analyze as much of each individual fruit as possible, as well as a sufficiently large number of fruits to obtain representative values for the metabolite contents of each cultivar. Here we individually analyzed 10 randomly chosen apples at each time point [harvest, 10 days of shelf life (SL), and 3 months of cold storage (CS)] for each cultivar. Apples were randomly selected

from the total crop, which was harvested from between 3 and 10 trees per variety, without any attempt being made to account for fruit canopy position. As summarized in **Table 1B**, the average percent standard deviations at harvest for the mean L-AA and GSH contents of each individual cultivar were 19.5 and 22.0%, respectively, which is comparable with the mean percent standard deviation for fruit fresh weight of 18.3% per cultivar (**Table 1A**). Overall, DHA contents at harvest varied between 0 and 25.1% of the total L-AA pool (L-AA + DHA), with a mean value of 12.1%. GSSG contents varied between 0 and 46% of the total GSH pool (GSH + GSSG), with a mean value of 12.9% (**Table 1C**). These mean proportions of DHA and GSSG indicate that overall the tissue was healthy and nonstressed and suggest that significant oxidation of the antioxidant pools did not occur during sample preparation.

Mean Intercultivar Differences. The mean fruit L-AA, DHA, GSH, and GSSG levels for the sample group at harvest are summarized in **Table 1B**,**C** and the individual values per cultivar in **Table 2**. The group mean total L-AA content (i.e., L-AA + DHA) was 722.1 nmol/gfw and varied between 401 and 1448 nmol/gfw. This corresponds to a maximal 3.6-fold variation in mean L-AA contents, which is smaller than first expected from the available literature (*11*). Interestingly, the maximum measured mean L-AA content of 1283 nmol/gfw is similar to the maximal value of 1450 nmol/gfw recently reported by Planchon et al. during their analysis of 30 "old" Belgian apple cultivars harvested in 1999 (*22*). Unfortunately, these authors did not report on DHA, GSH, or GSSG contents. Nonetheless, the results together suggest that the genetic potential for breeding for increased fruit L-AA contents is currently limited, even though *Malus* L-AA contents are relatively low in comparison to some other fruits (*11*). Mean GSH contents varied between 20.7 and 90.9 nmol/gfw, representing a 4.4-fold variation in concentrations. As far as we know this is the first report of the variability present in the fruit GSH content in *Malus*. These values for GSH content are approximately 2−3-fold lower than GSH contents in *Malus* foliar tissue and some 4-fold lower than seed GSH levels (*18*).

Relationships between the L-AA and GSH Pools. Plotting mean total L-AA (L-AA + DHA) content versus mean total GSH (GSH + GSSG) content of fruit from each of the cultivars examined here (**Figure 2A**) shows that fruits from cultivars with a higher mean total L-AA level tend to have a higher mean total GSH concentration. Although this is only a weak correlation across the group of 31 cultivars ($R^2 = 0.273$), the relationship between total L-AA and total GSH is much clearer when one is looking at the individual fruit of each cultivar (**Figure 2B**). Interestingly, though, within different parts of a single fruit there is an inverse relationship between L-AA and GSH levels, and areas with high L-AA tend to have lower GSH levels and vice versa (*18*).

Influence of Storage Conditions on Fruit L-AA and GSH Content. L-AA and GSH levels at harvest can be considered important markers of fruit quality and a reflection of a tissue's ability to withstand stress conditions. However, relatively few apples are consumed directly at harvest, so it was important to determine the influence of standard storage conditions on metabolite levels. The concentrations of L-AA, DHA, GSH, and GSSG were determined after cold storage (CS) (3 months at 1 °C) and shelf life (SL) (10 days at room temperature).

The overall group changes associated with storage under either CS or SL conditions are summarized in **Table 1**. **Table 1A** shows that there is no significant difference in the mean fresh weights of the fruits analyzed at each time point, indicating

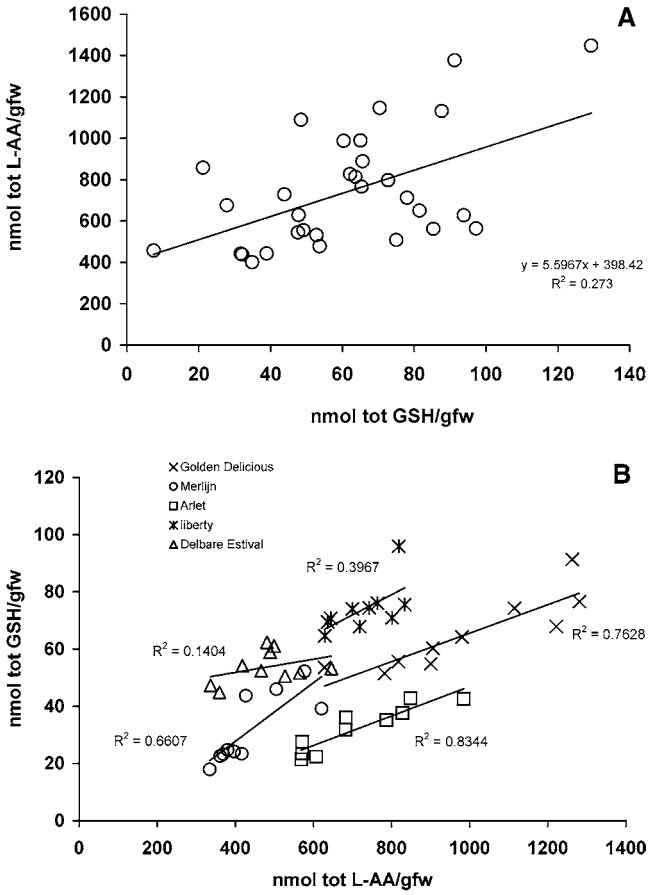

Figure 2. Relationship between mean total L-AA and mean total GSH contents: (**A**) mean values for all fruits of each individual *Malus* cultivar sampled at harvest; (**B**) 10 individual analyses for each of 5 different apple cultivars.

that any differences in metabolite concentrations are not related to changes in the fresh weights of the fruit. **Table 1B** clearly shows that SL storage results in a decrease in the group mean L-AA and total L-AA contents, with average losses of 32.4 and 35.1%, respectively. These differences are statistically highly significant ($P = 0.007$ and $P = 0.002$, respectively) but are not significantly different from the L-AA and total L-AA contents of fruit after CS. By comparison, GSH and total GSH levels were significantly different at all three time points measured ($P < 0.001$). Surprisingly, whereas GSH levels decreased by 16.7% under SL conditions, the mean GSH and total GSH levels actually increased after CS. The basis for the observed increase in GSH is not clear but may represent an acclimatory response of the fruit to the cold stress conditions, where it functions to maintain protein thiol groups in a reduced and active state. However, as noted above, this was not accompanied by an overall increase in fruit L-AA concentrations, which might have been expected on the basis of the response of other plant species during adapatation to cold (*23*, *24*). This lack of response by the L-AA pool may be due to the incapacity of mature apple fruit to synthesize its own L-AA (*18*). Interestingly, there were no significant changes in the mean oxidation status of either the L-AA or GSH pools under any of the storage conditions, suggesting that the alterations in steady state tissue concentrations during acclimation to each of the conditions generally did not lead to a net oxidative stress.

Sections **B** and **C** of **Table 1** also show the influence of storage on the fruit antioxidant concentration ranges and, therefore, also provide an idea of the range of biodiversity for

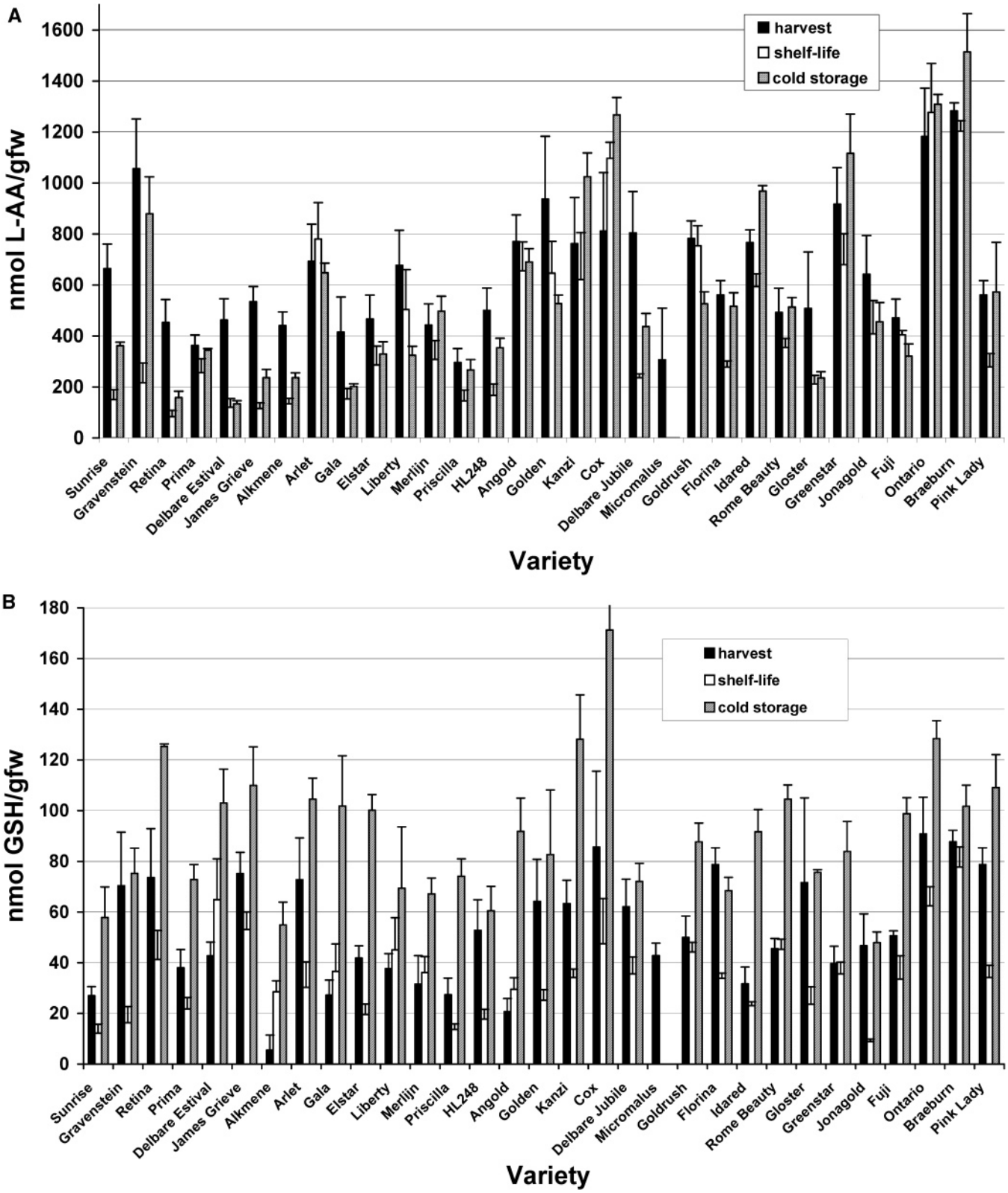

Figure 3. Changes in (**A**) mean total L-AA contents and (**B**) mean total GSH contents at harvest and after shelf life and cold storage conditions.

L-AA and GSH present in the sampling population. As can be seen, the maximal concentration differences in L-AA/total L-AA and GSH/total GSH levels at harvest are low, at ~4-fold. However, after SL the between-cultivar differences in L-AA/total L-AA concentrations increase to 12−15-fold and after CS to 10−11-fold, respectively. The between-cultivar differences in fruit total GSH concentrations by comparison showed a much smaller change in response to storage, increasing from 4.6-fold to a maximal 6-fold variation in after SL, although there is an overall increase in total GSH levels in response to cold. These results indicate that the differences between individual cultivars derive primarily from their ability to preserve L-AA pools, and furthermore, that the increase in variability in L-AA/total L-AA is mainly due to the loss of L-AA/tot L-AA by "low-vitamin C" cultivars. Overall, our results show that storage for 10 days at room temperature (SL) is likely to be more detrimental to

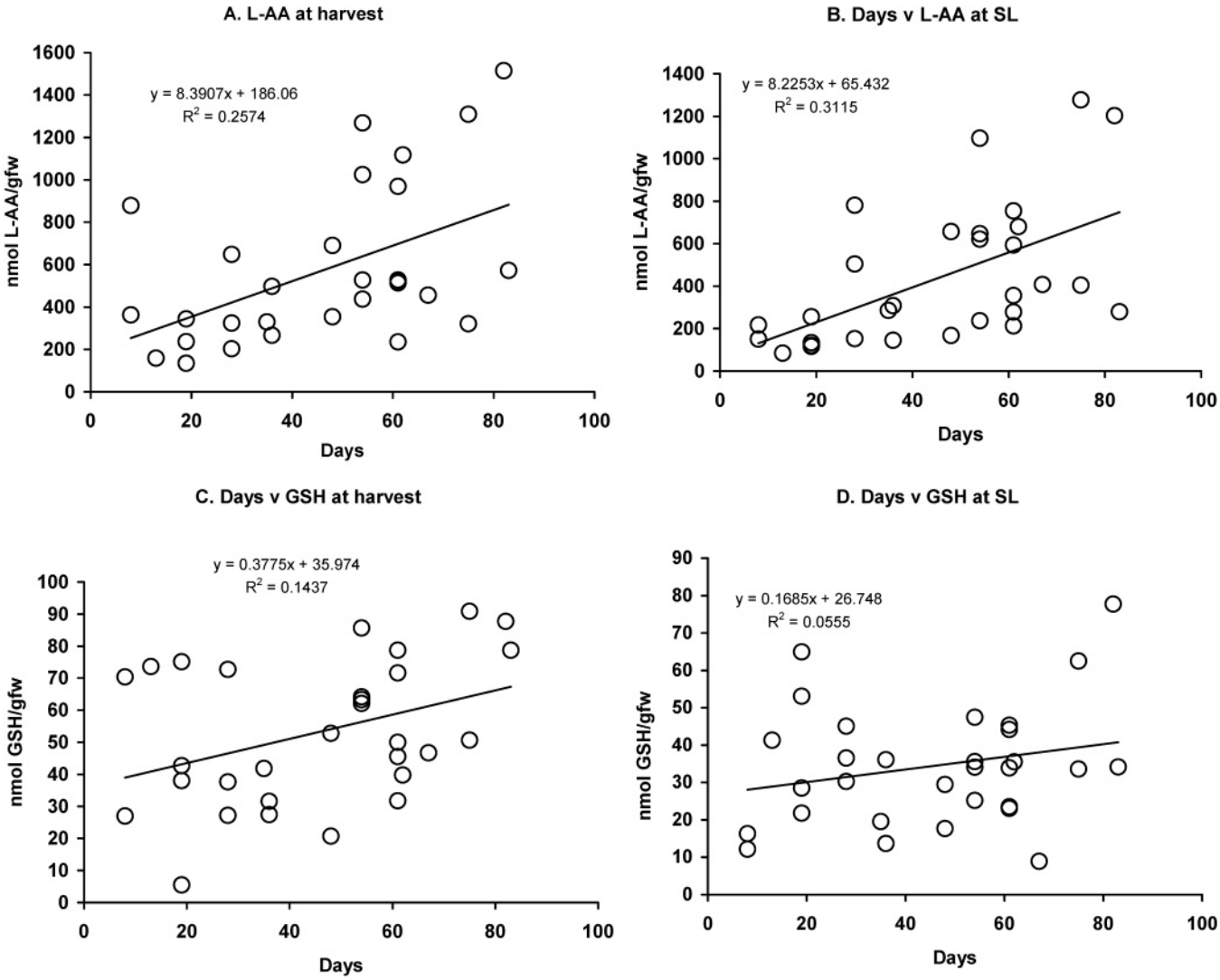

Figure 4. Influence of harvest date on fruit antioxidant contents: (**A**) L-AA at harvest; (**B**) mean L-AA at SL; (**C**) mean GSH at harvest; (**D**) mean GSH after SL. Days correspond to the date of harvest as the number of days after August 1, 2002.

fruit antioxidant (nutritional) levels than storage for 3 months at 1 °C and that there are substantial differences in the ability of cultivars to maintain their L-AA and GSH pools during storage.

Cultivar-Dependent Changes in Antioxidant Status during Storage. The L-AA/GSH contents of individual cultivars responded very differently to CS and SL, ranging from no significant change in L-AA/GSH to very heavy losses—summarized in **Figure 3**. Maximal losses of L-AA of ~80% following SL were observed for the "early" cultivars such as Sunrise and Gravenstein, and this was accompanied by losses of >50% of the GSH content. Both of these varieties are known to have poor storage qualities (e.g., maximum 1 month of CS), with susceptibility to browning and rot. For varieties such as Arlet and Angold, there was no significant difference between L-AA at harvest, SL, or CS, and Arlet can be stored for up to 6 months at 1 °C. For others such as Delbare Estival, any storage condition results in substantial losses of L-AA, but not necessarily in GSH content, and this variety shows intermediate storage behavior, maintaining quality for up to 4 months at 1 °C. Finally, cultivars such as Greenstar and Braeburn are able to maintain or actually slightly increase both L-AA and GSH levels under both CS and SL and can retain quality for up to 6 months at 1 °C. These results suggest that L-AA and possibly GSH content can serve as markers for storage quality—at least in certain cultivars—and may possibly be related to the capacity of the fruit to withstand long-term storage. The fact that certain varieties are able to slightly increase their L-AA (and GSH) contents in response to CS is suggestive of an acclimative response to low temperature (and other stresses), as has been observed in other plant tissues, but counteracts previous observations that mature apples are incapable of L-AA biosynthesis (*18*). The fact that this response—when present—is observed only in a few middle—late-ripening varieties (Cox, Idared, Greenstar, and Braeburn) suggests that this capacity could be related to the fruit climacteric. We have observed (M. W. Davey, unpublished observations) that young and developing fruits are capable of L-AA biosynthesis but that this biosynthetic capacity is gradually lost with maturation, suggesting that varieties that ripen slowly or are picked early could also conceivably maintain an L-AA biosynthetic capacity postharvest. However, this hypothesis still needs to be rigorously tested. Currently nothing is known about the capacity of fruit to synthesize or recycle GSH.

Interestingly, we see from **Figure 4A** that cultivars with the highest L-AA/total L-AA contents (e.g., Braeburn, Ontario) were always harvested late in the season, whereas the cultivars with the lowest L-AA/total L-AA contents were always among the early-ripening varieties (e.g., Prima, Retina). Although across the group harvest time is only weakly correlated with L-AA

and GSH levels (**Figure 4**), the results do allow us to identify those outliers or cultivars that perform better (or worse) than expected for the time of year, for example, in this season cv. Gravenstein, and the results suggest that as a general rule that cultivars with the highest fruit L-AA contents will be late-ripening varieties. It is presently unclear, however, from our data what role environment—and temperature in particular—plays in modifying fruit antioxidant contents. It is conceivable that the decrease in ambient temperature during the season is at least partially responsible for the higher L-AA contents observed in later varieties, if fruit L-AA contents decrease with increasing temperature. Overall, however, it is important to realize that during development, the fruit L-AA/GSH contents of an individual cultivar decrease with increasing fruit maturity (*18*).

Correlations between Antioxidant Content and Other Physiological Parameters. At each time point, fruit quality from each variety was assessed using standardized physiological measurements on each of 10 individual fruits. Such values are often used by producers to estimate the optimal harvest times of cultivars. The mean values for these parameters at harvest for each of the varieties examined here are summarized in **Table 2**. Examining the statistical correlations between the major physiological parameters measured (hardness, %Brix, mean titratable acid, weight) and mean L-AA/total L-AA and GSH/total GSH at harvest revealed a number of highly significant relationships ($P < 0.01$). Of these, the most important are between L-AA and GSH ($R = 0.4987$), mean percent Brix and total GSH ($R = 0.5003$), and percent Brix and L-AA ($R = 0.3256$). These correlations weakened after SL, so that the significance was still high ($P < 0.01$), that is, between L-AA and GSH ($R = 0.3130$), between mean percent Brix and GSH ($R = 0.3806$), and between percent Brix and L-AA ($R = 0.1913$). Correlations between L-AA/GSH and titratable acidity and hardness were even lower, meaning that the usefulness or the predictive value of these relationships is limited due to the low correlation coefficient, that is, the near horizontal slope of the correlation graphs.

In summary, from the available literature mean total L-AA contents (L-AA + DHA) in apple range from 114 to 1700 nmol/gfw (*11*, *25*), representing a 7.5-fold variation. However, in our sampling population, the variability at harvest in mean total L-AA was actually smaller, at only 3.6-fold, which we feel at least partially reflects the care taken during sampling, the standardized methodologies, and the short time required for analysis, all of which prevent L-AA breakdown. This relatively small variation at harvest suggests that it will be difficult to substantially increase L-AA contents via classical breeding techniques. However, the degree of variation in L-AA contents increased markedly after storage, indicating that in *Malus* the metabolism of L-AA postharvest is at least equally as important to the fruit antioxidant content received by the consumer as net values at harvest. This is perhaps not surprising as we have recently demonstrated that mature apple fruits are incapable of *de novo* L-AA biosynthesis (*18*) and are therefore entirely dependent upon phloem-derived L-AA, transported from the leaves (*26*).

Interestingly, there exists a weak correlation between harvest date and fruit L-AA contents, with the result that the highest L-AA contents are always found in late-harvested cultivars, and cultivars with the lowest contents are always among the early-harvested cultivars. In general, cultivars with a high L-AA content also have a high GSH content. Thus, simply on the basis of predicted harvest time, it may be possible to significantly enhance the probability of selecting for "antioxidant-rich" or "nutritionally enhanced" cultivars. It is unclear from our results, however, the extent to which these values are influenced by environment.

Finally, it seems that the combination of a high L-AA content, a high GSH content, and late ripening (or slow senescence) is associated with and possibly partially responsible for improved storage behavior in apple varieties. The observed 11−15-fold variation in mean total L-AA contents following storage also suggests that there is sufficient biodiversity within the breeding population of commercial cultivars to be able to breed for this characteristic but that selection should take place after storage.

ABBREVIATIONS USED

L-AA, L-ascorbate, L-ascorbic acid, vitamin C; DHA, dehydroascorbate, dehydroascorbic acid; GSH, glutathione, γ-glutamylcysteinyl glycine; GSSG, glutathione disulfide; CS, cold storage; SL, shelf life.

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