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Variation in Ascorbic Acid and Oxalate Levels in the Fruit of *Actinidia chinensis* Tissues and Genotypes

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Ascorbic acid and total oxalate were measured in fruit from six genotypes of *Actinidia chinensis*. Ascorbic acid was separated from oxalate in fruit extracts by HPLC and quantified from absorbance at 245 nm, whereas oxalate was measured enzymatically in the HPLC eluate. Levels of whole fruit mean ascorbic acid in the different genotypes ranged from 98 to 163 mg/100 g of fresh weight (FW), whereas mean oxalate varied between 18 and 45 mg/100 g of FW. Ascorbic acid was highest in the inner and outer pericarp, whereas oxalate was concentrated in the skin, inner pericarp, and seed. Essentially no ascorbic acid was found in the seed. Each tissue clustered separately when the tissue ascorbic acid and oxalate data were normalized to the whole fruit level of ascorbic acid and oxalate in that genotype and plotted against each other, suggesting that oxalate is not a sink for excess ascorbic acid but that oxalate formation is regulated.

KEYWORDS: Kiwifruit; vitamin C; oxalic acid; ascorbate; HPLC

INTRODUCTION

The genus *Actinidia* contains almost 75 species and about 120 taxa (A. R. Ferguson, personal communication) with large differences in fruit composition between species and often within species (*1*, *2*). Two species are extensively cultivated and marketed worldwide, *A. deliciosa* and *A. chinensis*. In China, 24% of the commercial kiwifruit plantings are *A. chinensis* (*3*), whereas outside China, the only significant quantity of *A. chinensis* fruit produced is of cv. Hort16A (*4*), which is becoming more familiar to the consumer owing to its sweet, tropical flavor and high ascorbic acid content, up to 50% higher than that in cv. Hayward (*5*).

A range of values for ascorbic acid, an important health component of kiwifruit, have been reported (*6*, *7*). Ascorbic acid content of fruits of *A. deliciosa* range from 30 to 400 mg/100 g of fresh weight (FW) (*2*), whereas for the cultivar Hayward the reported range is 80−120 mg/100 g (*7*). Relatively high concentrations of ascorbic acid are also reported in fruit of *A. arguta*, *A. chinensis* (*5*, *8*), *A. chrysantha*, and *A. polygam*, with very high levels in *A. eriantha*, *A. latifolia* (>1% FW) (*2*), and *A. kolomikta* (*9*). Ascorbic acid is determined according to a variety of established methods including colorimetric titration (*10*), enzymatic assays based on ascorbate peroxidase or oxidase (*11*, *12*), electrochemical methods (*13*−*15*), and HPLC coupled with electrochemical detection (*13*, *16*) or by HPLC coupled with UV detection (*13*, *17*).

Oxalate is present in plants in soluble (oxalic acid, as well as soluble potassium and sodium oxalate) and insoluble forms (largely as calcium oxalate crystals) (*18*). It is often suggested to be a breakdown product of ascorbic acid (*19*, *20*), although in spinach leaves, glycolate was shown to be more efficient as a precursor of oxalate than ascorbic acid (*21*). Oxalate has adverse nutritional effects, binding Ca^{2+}, Mg^{2+}, and Fe^{2+} and lowering their bioavailability in the diet by decreasing their absorption (*22*). High levels of oxalate in the body, resulting from the ingestion of a high-oxalate diet and from endogenous metabolism, may result in calcium oxalate crystallization and kidney stone formation (*22*, *23*). The fine needlelike calcium oxalate raphide crystals found in kiwifruit irritate the mucous membranes in the mouth (*24*, *25*).

Oxalate is frequently quantified in plant tissue by HPLC using UV absorption at 210−224 nm (*24*, *26*−*28*), although other compounds also elute at similar retention times and interfere with oxalate estimation. Alternatively, oxalate is measured directly in plant tissue extracts using enzymatic coupling (*29*, *30*), but these enzymatic methods can be severely interfered with by ascorbic acid and other plant components (*29*, *31*), and reliable oxalate estimates are rare. To avoid this interference, the oxalate may be separated from the ascorbic acid by HPLC and oxalate then measured enzymatically (*18*) using a specialized enzyme reactor system. This method gave values for *A. deliciosa* (then classified as *A. chinensis*) total oxalate of 23.0 mg/100 g of FW (*18*).

In this work we report a modified method of the enzyme reactor method (*18*) for determining oxalate and ascorbic acid levels in a single extract of kiwifruit and report ascorbic acid and oxalate levels in fruit tissues of a series of *A. chinensis* genotypes. We establish that ascorbic acid and oxalate levels in fruit tissues are not necessarily related and suggest that if oxalate is a breakdown product of ascorbic acid, then this process is highly regulated.

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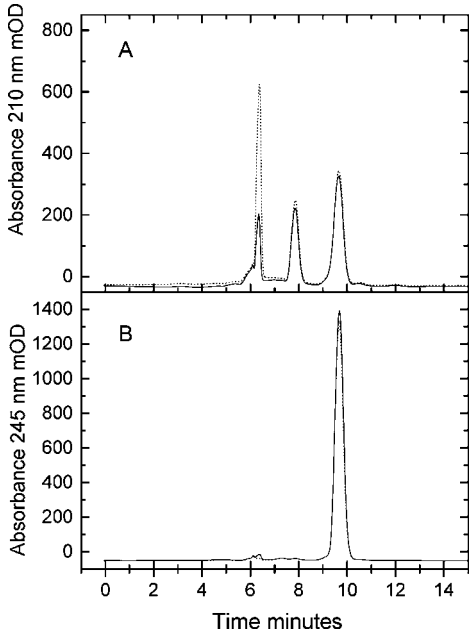

Figure 1. HPLC chromatogram for the separation of oxalate (**A**; RT 6.2 min) and ascorbic acid (**B**; RT 9.6 min) using a 7.8 × 300 mm Aminex HPX-87H HPLC column (Bio-Rad) with 2.8 mM H_2SO_4, as the mobile phase, at a flow rate of 0.6 mL/min. Solid lines represent kiwifruit extract without added extra oxalate and dotted lines, with 1.6 μmol of added oxalate. Absorbance was measured as mOD units.

MATERIALS AND METHODS

Samples. Ripe fruit samples were obtained from six genotypes, each from a separate accession of *A. chinensis* seed from China: CK64_12 and CK65_07 (open-pollinated seed from two female selections from Henan); CK66_02 (open-pollinated seed of cv. Lushanxiang, a selection from Jiangxi that came from Beijing); CK67_03 (open-pollinated seed from a sport of cv. Jin Niu 2 in Henan); CK68_09 (open-pollinated seed from cv. Caoying 1, which came from Shaanxi); CK69_05 (open-pollinated seed from cv. Lantain 1, which came from Shaanxi). Vines were grown at Te Puke, New Zealand. Fruit samples were picked from the vines and processed within 24 h. One centimeter equatorial cross sections of the fruit were separated into skin, outer pericarp (OP), and inner pericarp including seeds (IP) and core. The inner pericarp was defined as that region containing locules and is the predominant tissue in kiwifruit. Tissues from six fruits were pooled. In addition, six whole fruits were individually sampled and stored. All samples were immediately frozen in liquid N_2 and stored at −80 °C.

Extraction and Quantification of Ascorbic Acid and Oxalate. Each frozen tissue sample was ground to a fine powder in a Cryomill at liquid nitrogen temperature. About 200 mg of frozen powdered tissue was suspended in 4 volumes of 0.5 N HCl containing 4 mM TCEP (Pierce), vortexed for 20 s, and incubated in a heating block for 2 h at 40 °C. We chose to use TCEP, in the extraction solution, because it is a more effective reducing agent under acidic conditions (*32*) than DTT. The extract was centrifuged at 4 °C, and 20 μL of the supernatant was injected onto a 7.8 × 300 mm Aminex HPX-87H HPLC column (Bio-Rad). The column was run with 2.8 mM H_2SO_4, at a flow rate of 0.6 mL/min, and the amount of ascorbic acid was calculated from absorbance at 245 nm [retention time (RT) 9.6 min], using ascorbic acid (Sigma, St. Louis, MO) as a standard. The peak was authenticated as ascorbic acid by showing that it was completely degraded by ascorbate oxidase at pH 5.5.

The oxalate peak (RT 6.2 min) was well separated from the ascorbic acid peak (**Figure 1**), and a fraction between 6 and 7.5 min was collected free of ascorbic acid. The HPLC oxalate peak fraction was stored at −20 °C until assay. Oxalate was assayed in 96-well clear microtiter plates (Greiner Bio-One GmbH). Standard oxalate (0.4−3.2 nmol of oxalate/well) or sample, made up in 100 μL of 2.8 mM H_2SO_4, was added to 100 μL of color reagent (100 mg of DMAB and 9.5 mg of MBTH dissolved in 2 mL of methanol, the volume made up to 200 mL with 0.1 M citrate buffer containing 7 mM EDTA, pH 3.2, filtered through 0.45 μm cellulose acetate membrane and stored in the dark at 4 °C). After an initial absorbance reading of the plate at 600 nm, 4 μL of oxalate oxidase/horseradish peroxidase mixture (Oxalate reagent B, Sigma catalog no. 591-2) was added to each well, the plate was incubated for 25 min at 30 °C, and the absorbance was read again. Oxalic acid dihydrate (Sigma) was used as standard, and the final results were corrected to anhydrous oxalic acid.

Recoveries of 96 ± 3% (means ± standard errors, $n > 5$) for oxalate and 98 ± 2% for ascorbic acid were measured when different tissues of a range of different genotypes were spiked with different amounts of oxalate or ascorbic acid (0.5−2 times the endogenous levels) prior to homogenization. Recoveries were independent of the amount of added oxalate.

RESULTS AND DISCUSSION

We have modified an existing method to measure oxalate (*18*) and used it to determine oxalate and ascorbic acid in the same sample. This avoids the potential interference of ascorbic acid and other compounds on the measurement of oxalate (*33*) and gives excellent recoveries. The original method used an HPLC on-line enzyme reactor involving the use of an amperometric detector (*18*), but our modified method achieves similar results without the need for a specialized HPLC detector, although it would probably cost more to assay the oxalate through consumption of oxalate oxidase. The oxalate and ascorbic acid were clearly separated from each other (**Figure 1**), recoveries of added oxalate were close to 100%, and we avoided the problem of estimating oxalate from absorption at 210 nm in the presence of other absorbing compounds that eluted close to the void volume of the column.

We used relatively mild acidic conditions for extraction to minimize oxalate generation (*18*) and to minimize formation of potential interfering polyphenols. Although oxalate and especially ascorbic acid contents of kiwifruit have been reported separately many times (*6*, *7*, *34*−*39*), there appear to be few studies (*24*, *40*−*43*) that measured both compounds simultaneously in *A. deliciosa* fruit and, to the best of our knowledge, none in *A. chinensis*.

Soluble and Insoluble Oxalate. Many papers report both soluble and insoluble oxalate as separately measurable components of the total oxalate pool (*18*, *24*, *39*, *40*). Initially we investigated measuring both components, but rapidly came to the conclusion that because the equilibrium between the two forms of oxalate depends on the pH, the Ca^{2+} ion concentration, and time, the relative levels of soluble and insoluble oxalate will depend on extraction conditions. Kiwifruit, for example, is an acid fruit, with a pH of ∼3 when extracted in water (data not shown). Typically extracts of leaves and less acid fruits have pH values as high as 5. The pK_s for Ca oxalate is 8.57, and the pK_a values for the protonation of oxalate (pK_{a1} = 1.46, pK_{a2} = 4.40) are very relevant to typical extraction conditions. Reported estimates of soluble oxalate in kiwifruit were up to 38.5 mg/100 g of FW in *A. deliciosa* Hayward (*24*, *39*, *40*, *43*), whereas the best estimate of soluble oxalate, by the enzyme reactor system, is 0.8−3.4 mg/100 g of FW in *A. deliciosa* (then classified as *A. chinensis*) (*18*). A trace of soluble oxalate was reported in peeled fruit of *A. deliciosa* (similarly then classified as *A. chinensis*) after ethanol extraction and gas chromatography (*44*). After various tests of extraction in which we varied the pH in the extraction solution, we found that the amount of soluble oxalate extracted was very dependent on the methods used, although the insoluble oxalate was always the bulk of the total oxalate pool (data not shown), in accordance with the

Table 1. Total Oxalate and Ascorbic Acid in Whole Fruit of Six Genotypes of *A. chinensis*

A. chinensis genotype	total oxalate (mg/100 g of FW ± SE)	ascorbic acid (mg/100 g of FW ± SE)	ascorbic acid/ oxalate ratio
CK64_12	26 ± 2	98 ± 2	3.7
CK65_07	45 ± 3	109 ± 1	2. 4
CK66_02	36 ± 1	163 ± 1	4.5
CK67_03	36 ± 2	155 ± 2	4.3
CK68_09	20 ± 2	109 ± 2	5.6
CK69_05	18 ± 1	120 ± 2	6.7

findings of Hönow and Hesse (*18*). Consequently, we decided to optimize the method to extract total oxalate and not to separate the soluble and insoluble components of oxalate.

Ascorbic Acid and Oxalate in Whole Kiwifruit. Ascorbic acid in the whole fruit of the six genotypes investigated varied between 98 and 163 mg/100 g of FW (**Table 1**) in comparison to 80−120 mg/100 g of FW reported for the most popular *A. deliciosa* Hayward fruit (*7*). There are few reports on ascorbic acid in *A. chinensis*. Cv. Jin Nong 1 is reported to contain114.6−158.4 g/100 g of FW (*8*) and Hort16A to contain up to 50% more vitamin C than *A. deliciosa* Hayward (*5*).

In this work, total oxalate varied 2.5-fold in different genotypes (**Table 1**). Literature values for oxalate in *A. chinensis* are rare. Total oxalate of 25.4, 26.1, 23.0, and 14.0 mg/100 g of FW have been reported in mature fruit of the *A. chinensis* cultivars Golden King and Yellow Queen (*39*). The variability for oxalate (calculated as the ratio of the standard error to the mean) within cultivars averaged 4.7 times higher than for ascorbic acid, whereas the absolute standard errors for oxalate and ascorbic acid were similar (**Table 1**).

Oxalate has been hypothesized to be derived from ascorbate (*19*, *20*), and so it might be expected that there should be a relationship between the two. However, a plot of ascorbic acid in whole fruit against oxalate gave a slope close to zero of 0.40 ± 0.17 ($n = 129$, $p < 0.023$) with the linear regression explaining little of the variation in the data ($r^2 = 0.04$) (data not shown). The lack of a relationship between ascorbic acid and oxalate, in whole fruit, is illustrated by the two genotypes with ascorbic acid levels around 110 mg/100 g of FW (CK65_07 and CK68_09), where oxalate varied from the highest to almost the lowest values measured. Thus, any substrate−product relationship between ascorbic acid and oxalate levels is not obvious in whole fruit (**Table 1**).

Tissue Distribution of Oxalate and Ascorbic Acid. The outer and inner pericarp contained comparable and relatively high levels of ascorbic acid similar to those in whole fruit (**Figure 2**). Values ranged from 91 mg/100 g of FW for CK64_12 to 194 mg/100 g of FW for CK66_02, in comparison with 34 and 89 mg/100 g of FW for the core and skin. In contrast to our data, others, using histochemical staining, have reported that ascorbic acid was high in the IP but low in the OP of *A. deliciosa* fruit (*40*), whereas uniform vitamin C distribution was reported in skin, OP, IP, and core of Hayward kiwifruit (41.7, 42.9, 45.5, and 42.3 mg/100 g of FW, respectively) (*35*).

The core and OP contain the lowest concentrations of oxalate (<20 mg/100 g of FW and sometimes undetectable levels), whereas the skin and IP contain much higher levels (**Figure 2**). To the best of our knowledge, there have been no previous reports of oxalate in individual tissues of the kiwifruit. There is greater variation in the levels of oxalate between genotypes within a tissue than there is for ascorbic acid, especially for the OP and core (**Figure 2**). For example, the variations in the

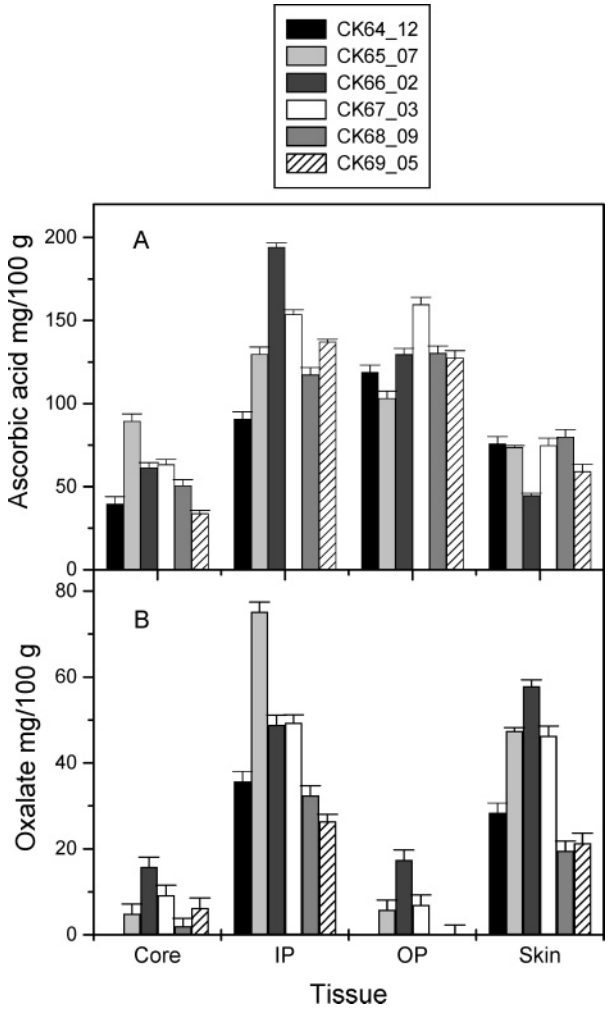

Figure 2. Ascorbic acid (**A**) and oxalate (**B**) concentrations in different tissues of six genotypes of *A. chinensis*. Error bars denote 0.95 confidence intervals. Apparent missing columns had zero oxalate.

average core and OP oxalate values across genotypes were found to be 2.5- and 9-fold higher than the variation in the average ascorbic acid values, respectively.

There was no significant correlation within tissues across different genotypes between ascorbic acid and oxalate levels (data not shown). However, when the tissue levels of oxalate and ascorbic acid in a given genotype were normalized to the average for that substance and genotype, the six genotypes formed separate clusters for the four tissue types (**Figure 3**; note the clusters were still present in non-normalized data). These data were analyzed by multivariate analysis of variance and tested the hypothesis of there being no difference among tissues. This overall hypothesis is rejected as tissue differences were found to be significant ($p < 0.0001$) as indicated by Wilk's lambda and other multivariate test statistics. Thus, for oxalate, only the cortex and the outer pericarp were not significantly different. For ascorbic acid, the outer pericarp was not significantly different from the inner pericarp, and the cortex was not significantly different from the skin. These data are consistent with the hypothesized conversion of ascorbic acid to oxalate being regulated and different tissues having different rates of conversion.

The IP contains the seeds, and so we tested both seed and flesh tissue of the IP to establish where the ascorbic acid and oxalate were in the IP of CK64_12. The seeds contained 66 mg of oxalate/100 g (compared to 36 mg/100 g of FW in the

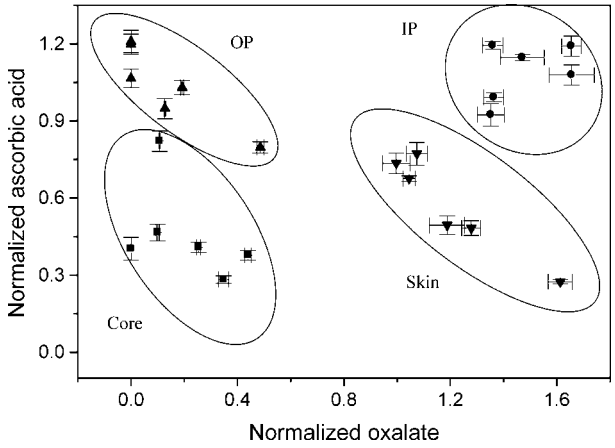

Figure 3. Normalized ascorbic acid and oxalate contents of different tissues of six genotypes of *A. chinensis*: (●) IP; (■) core; (▲) OP; (▼) skin (the data points for each tissue type are enclosed). Normalized ascorbic acid was calculated as the ratio of ascorbic acid in a tissue sample for a genotype to the average WF ascorbic acid found in that genotype. Normalized oxalate was calculated as the ratio of oxalate in a tissue sample for a genotype to the average WF oxalate found in that genotype. Standard errors for the means are also shown (*n* between 2 and 30).

IP), but little ascorbic acid was detected (2.8 mg/100 g of seed weight), even after reduction with TCEP (*32*). Oxalate extracted from unground seeds was 41 mg/100 g, which indicates that most of the oxalate may be in the outer cell layers, probably in the seed coat or testa. Note that the seed has a higher dry weight to FW ratio compared to other fruit tissues. The oxalate was not extractable with water and therefore is of the insoluble form. However, microscopic examination of the seeds revealed no obvious crystals of the form found in the soft tissue of the fruit. It is possible that oxalate is present in seeds in the form of small crystal sand as found in *Vitis vinifera* seeds (*45*). When we divided the IP into an outer zone without seeds and an inner zone with seeds, we found that the outer zone contained only 23 mg of oxalate/100 g of FW, whereas the inner zone contained 44 mg/100 g. This suggests that the oxalate is concentrated in the seeds with lower levels in the fruit tissue.

In conclusion, the distribution of oxalate in fruit points to the protective role of oxalate in fruit against herbivory by insects and other organisms. High levels of oxalate in the skin and the seeds would deter animals from eating the fruit and the seeds, although as the seeds did not contain raphides, it cannot be the long crystals that protect the seeds. The clear clustering of normalized ascorbic acid versus normalized oxalate between tissues in kiwifruit suggests that the conversion of ascorbic acid to oxalate as well as oxalate turnover must be strongly regulated in different fruit tissues. As oxalate and ascorbic acid concentrations can be separated into tissue clusters, with high ascorbic acid and low oxalate found in the OP, and different cultivars show different ratios of ascorbic acid to oxalate (**Table 1**), it may be possible to select for high vitamin C without concomitant high levels of oxalate. Thus, screening of existing cultivars and of new selections for low oxalate and high ascorbic acid is being undertaken.

ABBREVIATIONS USED

A., *Actinidia*; DMAB, 3-(dimethylamino)benzoic acid; DTT, dithiothreitol; IP, inner pericarp; OP, outer pericarp; MBTH, 3-methyl-2-benzothiazoline hydrazone; TCEP, tris[2-carboxyethyl] phosphine hydrochloride; FW, fresh weight; WF, whole fruit.

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