

Description of Lignified Vascular Nodules in Fruit of ‘Gala’ and Other Apple Cultivars

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Abstract. Nodules associated with the main cortical vascular bundles in fruit of the ‘Gala’ apple (*Malus × domestica* Borkh.) strains ‘Royal’ and ‘Regal’ were observed in several growing seasons. The nodules were found in 68% (n = 586) of the fruits examined, with a mean of 2.5 nodules per fruit. The nodules were first detected in developing fruit 2 months after bloom and were normally 1–2 mm in diameter by commercial harvest maturity. The nodules, like the vascular bundles, were pale green. They were inconspicuous at first, but became conspicuous and unattractive and changed to brown or red as the fruit became overmature. Nodules in the fruit of the ‘Gala’ x ‘Splendour’ hybrid ‘8S 27-2’ were dark brown or red at picking maturity, and occurred with high frequency. Nodules were also observed in ‘Splendour’, but were small, pale green, and infrequent in this cultivar. Microscopic examination of the nodules revealed that they typically contained a central cavity surrounded by a lignified wall with small pigmented cells outside the wall adjacent to the cortex. Low-frequency irrigation cycle times generally promoted the development of nodules in both ‘Gala’ strains but nitrogen treatments did not affect nodule frequency in ‘Royal Gala’. Mean fruit nodule frequency tended to be higher, overall, in ‘Regal Gala’ (3.9) than in ‘Royal Gala’ (1.4).

Fruit quality defects, resulting from vascular bundle growth, development, and lignification, often described as stringiness or veininess, occur in several horticultural crops, including peach [*Prunus persica* (L.) Batsch] and apricot (*Prunus armeniaca* L.). Peach vascular bundles can accumulate anthocyanins and appear red, but degradation during cooking results in a change to brown and reduces quality of canned fruit (Hsia et al., 1965). If significant water stress occurs during fruit development, apricot develops prominent vascular bundles, which reduce quality of fresh and canned fruit (personal observation, W.D.L.).

No references are known in which nodules associated with vascular bundles have been described in apple fruit, but cell proliferation associated with necrosis of vascular bundles has been observed in development of apple fruit corking disorders (Simons, 1968; Simons et al., 1971). The vascular anatomy of apple fruit has been described in a detailed report by MacArthur and Wetmore (1939). The main vascular bundles of most cultivars are readily apparent when the fruit is serially sectioned

longitudinally, the largest bundles occurring near the core line (Zielinski, 1955). They are pale green in immature fruit, but they lose chlorophyll as the fruit ripens, becoming yellow, then light or dark brown in overmature fruit. Cultivars such as ‘Rome Beauty’ and ‘Regal Gala’ develop red vascular bundles when fruit development continues past commercial harvest time.

Apple cultivars have genetically determined differences in vascular bundle anatomy. Yang et al. (1995) compared stem tissue of nine spur-type cultivars with that of two non-spur cultivars, using ultrathin sections. The length and diameter of the vessel members of spur-type cultivars, as a group, were less than those of the non-spur types, and the spur types had more sieve elements.

Environmental stresses can promote excessive growth and development of vascular bundles in several crops, resulting in reduced quality. Nutrient and soil moisture stresses contributed to tomato (*Lycopersicon esculentum* L.) fruit veininess, a quality defect in whole, processed tomato, and the amount of vascularization was similar in three cultivars (Saunders et al., 1996). Salt stress and calcium ions acted synergistically to enhance lignification and secondary thickenings of the phloem cell walls of *Phaseolus vulgaris* L., as well as hastening and increasing lignification of the secondary thickenings and stringiness of the beans (Cachorro et al., 1993). Water deficit stress applied to sweet potato [*Ipomoea batatas* (L.) Poir.] had a similar effect in enhancing lignification of secondary tissues derived from vascular cambium (Ravi and Indira, 1996).

Stress treatments enhance metabolic activity, such as protein synthesis and cell proliferation, in cells associated with vascular bundles. In almond [*Prunus dulcis* (Mill.) D.A. Webb] fruit, mRNAs encoded by genes for proteins, such as extensin or alpha-tubulin, accumulated in dividing cells surrounding vascular tissues of the fruit (Garcia-Mas et al., 1996). The beta-glucuronidase (GUS) gene, driven by a heat shock protein promoter from soybean (*Glycine max* L.), resulted in heat-inducible protein expression levels that were highest in the vascular tissue (Prandl et al., 1995). When a fragment of the tas 14 dehydrin gene was fused to the GUS gene, it drove abscisic acid and osmotic stress induced GUS expression localized mainly in the vascular tissue, outer cortex, and root meristems of tobacco (*Nicotiana tabacum* L.). The tas 14 gene accumulates in response to mannitol, sodium chloride, or abscisic acid treatment. This coincided with the location of tas 14 gene expression in salt-stressed tomato (Parra et al., 1996).

Our report describes the form and occurrence of previously undocumented apple vascular nodules, which were found closely associated with cortical vascular bundles. The nodule frequency in several cultivars of apple grown in different locations was investigated over several growing seasons. The nodules were examined visually and microscopically. We also examined the nodule frequency in fruit samples of ‘Royal Gala’ and ‘Regal Gala’ from experimental plots with several nitrogen fertilizer and irrigation frequency treatments. Although nodules in fruit of these ‘Gala’ strains were inconspicuous at harvest, in overmature fruit of ‘Gala’ and some other cultivars, the nodules were conspicuous because of their dark color and, when apparent, were cause for rejection. Conspicuous, dark nodules would be expected to reduce quality of apples used in fresh-cut produce and some processed apple products, as well as in markets where fruit are sliced for display before consumption.

Materials and Methods

Apples used in these experiments were obtained from plots at the Pacific Agri-Food Research Centre (PARC) in British Columbia, Canada. Fruit of ‘Royal Gala’ and ‘Regal Gala’ were randomly picked from trees on M.7 rootstock in their eighth leaf in the cultivar repository, or from trees on M.7a rootstock in their sixth leaf in cultivar test plots. ‘Splendour’ fruit were from a single tree in its 13th leaf, grown on M.7 in the cultivar repository, and fruit of ‘8S 27-2’ were from three trees in their seventh leaf, grown on M.9 in two cultivar test plots. ‘8S 27-2’ is a hybrid of ‘Gala’ x ‘Splendour’ from the PARC breeding program. Fruit samples were also obtained from the experimental treatment plots described below. Fruit, selected randomly from the trees, were picked at commercial harvest maturity unless otherwise indicated. All the trees had been managed following recommended commercial practices and were in good health.

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Description of the vascular nodules. Samples of nodules were prepared for counting, examination, and photography by slicing apples longitudinally, then removing thin slices, 2–3 mm thick, sequentially from the exposed surface using a sharp knife. Large, longitudinally oriented vascular bundles, aligned with the ribs of the apple, were located at about the core line, midway through the cortex. The nodules were visible through the surrounding cortex because of their darker color, allowing the remaining cortex to be carefully removed, thus exposing the nodule. Slices of the whole apple were prepared for photography in this way, placed on a light tray illuminated from the rear, and photographed. Nodules photographed at higher magnification were prepared in the same way, but the final dissection was done with the aid of a stereoscopic microscope. They were then photographed using the camera attached to the microscope.

Microscopic sections were prepared by isolating a nodule with some adhering cortical tissue, giving a cube $\approx 3 \text{ mm}^3$. Samples were fixed in a solution of formalin, acetic acid, 95% alcohol, and distilled water (2:1:10:7, v/v). The fixed tissue was dehydrated in a series of t-butyl alcohol, embedded in Paraplast® (Sherwood Medical, St. Louis), and cut radially into 10- μm sections. Sections, attached to slides, were then stained with crystal violet and erythrosin B (Clark, 1981). This solution stained non-lignified cell walls red, and lignified walls violet. Photomicrographs were taken using bright field illumination.

The enzyme solution, UltraSP-L (Nova Nordisk Ferment Ltd., Dittingen, Switzerland), a macerating enzyme preparation that contains pectinase, cellulase, and hemicellulase activity but is not active against lignin, was used to separate the nodules from the cortex and to facilitate examination of their structure and composition. Nodules, prepared in this way, were examined with a stereoscopic microscope, dissected, and prepared as squashes for light microscope examination. This enzyme is often used to increase juice yield when processing apples.

Expt 1. Nodule frequency in fruit of 'Gala' and other cultivars. In 1994, 10 apples were examined from each of 10 trees of both 'Royal Gala' and 'Regal Gala'. Two trees of each strain were from the cultivar repository and eight from the cultivar test plot. In 1998, 20 apples of 'Splendour' were collected and nodules counted. Nodules were also counted in 16 apples of '8S 27-2'.

Expts. 2 and 3. The effects of nitrogen and irrigation on nodule frequency. Ten apples of 'Royal Gala' were collected from each plot from two experiments in 1992. The first (Expt. 2), was a 2×2 factorial with two levels of nitrogen fertilization, 80 and $200 \text{ kg} \cdot \text{ha}^{-1}$, and two weed control treatments, either complete control using herbicide or minimal weed control. The treatment plots were replicated five times. The second (Expt. 3), was a $2 \times 3 \times 2$ (method \times cycle time \times strain) factorial with either drip or microsprinkler irrigation emitters and three irrigation treatments in which

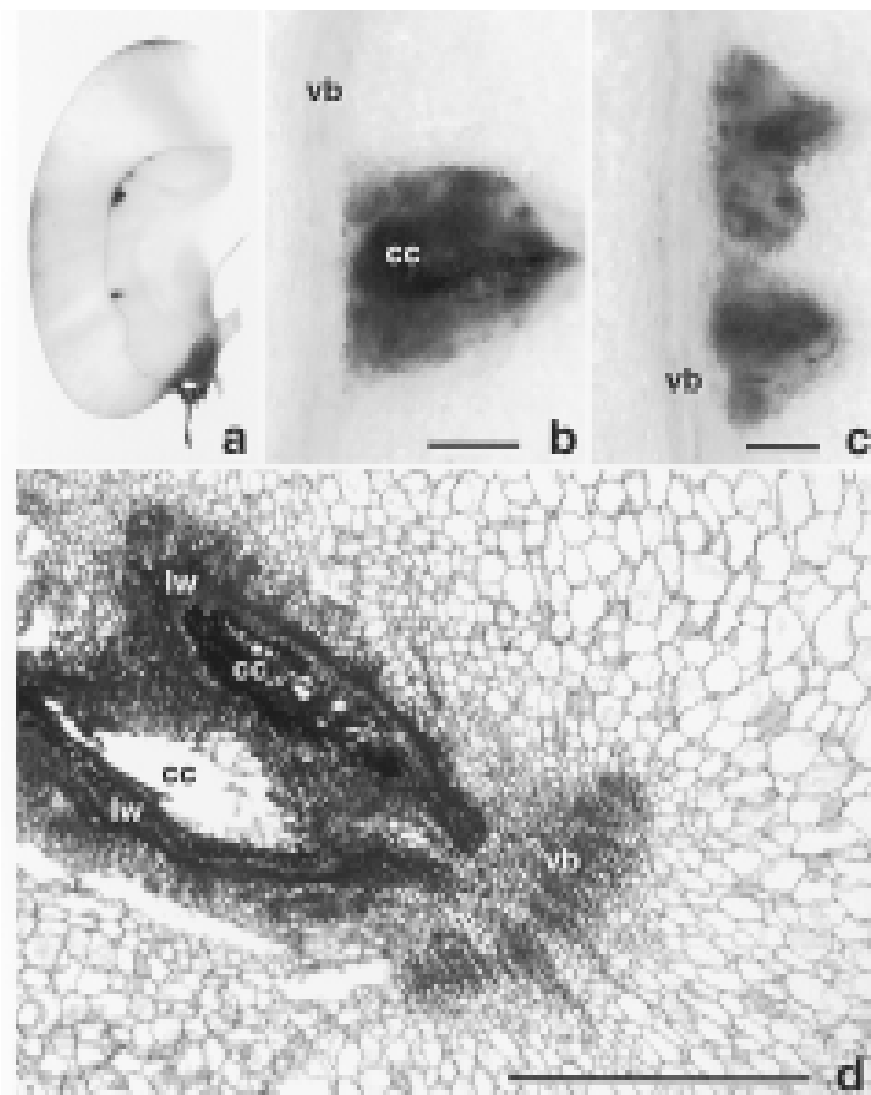


Fig. 1. (a) A hand section, 1–2 mm thick, of 'Regal Gala' apple fruit flesh with four nodules associated with a main vascular bundle; photographed with back illumination. (b) A hand section of a nodule associated with a vascular bundle (vb) illustrating the conical shape and the central cavity (cc) often present; '8S 27-2'; bar is 1.0 mm. (c) Three nodules associated with the vascular bundle (vb); '8S 27-2'; bar is 1.0 mm. (d) Section through a nodule and surrounding cortex with associated vascular bundle (vb) in cross section; central cavity (cc) and lignified wall (lw), stained with crystal violet and erythrosin; 'Royal Gala'; bar is 1.0 mm.

the same total amount of water was applied to each treatment plot, but the irrigation cycle was timed to result in high, medium, or low frequency of irrigation. The total water applied per cycle was 16 L/tree for the drip emitters and 36 L/tree for the microsprinklers. Trees were watered daily (high frequency), at 7- to 10-d intervals (medium frequency), or at 14-d intervals (low frequency). The plots in this experiment were split, with two replicates of each treatment planted to 'Royal Gala' and two to 'Regal Gala'. A more detailed description of these experiments was published previously (Nielsen et al., 1995, 1999).

For statistical analysis, the data were transformed to $\log(\text{count} + 1)$ to provide homogeneous variances. The effects of cultivar, nitrogen, and irrigation frequency treatments on the frequency of nodules per fruit were determined by using analysis of variance on the transformed data (SAS Institute, 1989).

Results

Description of the vascular nodules. A hand-sectioned slice of apple 1–2 mm thick revealed four nodules associated with the primary vascular bundle (Fig. 1). Three of the nodules were located toward the stem end where most nodules occurred. The nodules were invariably located on the core side of the associated vascular bundle. Additional hand sections, photographed using a stereoscopic microscope, showed that nodules were always closely associated with vascular bundles (vb) (Fig. 1 b and c). Also apparent is the generalized conical shape of the nodules, typically with a cavity in the center (cc), surrounded by lignified tissue (lw), although not all nodules developed a well-defined cavity. After digestion of the nodules in a solution of macerating enzyme, the lignified tissue at the center of the nodules remained undigested, was noticeably

hard when dissected, and was brown. Cutting a nodule in a plane perpendicular to the longitudinally oriented vascular bundle showed the lignified wall surrounding the two central cavities, the small cortical cells, which, before slide preparation, contrasted in color with the cortex, the vascular bundle in cross-section, and the normal cortex cells of the apple flesh (Fig. 1d).

Nodule frequency in 'Gala' strains and other cultivars. 'Royal Gala' and 'Regal Gala' had a mean of 2.06 (SE = 0.16) nodules per fruit. Analysis of data from the comparison of 'Gala' strains showed that the variation between trees was greater than the difference between strains, and that the difference between strains was nonsignificant ($P = 0.08$). 'Royal Gala' and 'Regal Gala' had mean nodule frequencies per fruit of 1.59 (SE = 0.20) and 2.53 (SE = 0.26), respectively. Data from Expt. 3 indicated a significant three-way interaction between strain, irrigation deficit, and emitter type ($P = 0.02$), preventing a direct comparison of the strains (Table 1). We noted that the mean nodule number for 'Regal Gala', as in Expt. 1, exceeded the number for 'Royal Gala' in every treatment plot.

Examination of '8S 27-2' showed that all 16 fruit contained nodules. The lowest frequency was five nodules per fruit, the greatest 34, with a mean of 11.5 (SE = 7.2). We consistently observed nodules in the fruit of this 'Gala' × 'Splendour' hybrid, and in most of the other progeny from this cross. This family of seedlings developed nodules regardless of location or growing season.

In contrast, examination of 'Splendour' showed that only four of the 20 fruit examined each contained one small, pale green nodule.

The effect on nodule frequency of nitrogen and irrigation frequency treatments. Nitrogen nutrition status with little or extensive weed competition (Expt. 2) did not influence nodule number of 'Royal Gala' (nitrogen effects, $P = 0.75$; weed control effects, $P = 0.91$). In this experiment, the mean number of nodules per fruit was 1.15 (SE = 0.33) (Table 2). The irrigation experiment (Expt. 3) showed a significant three-way interaction ($P = 0.02$). Emitter type had a small effect, but frequent irrigation, in general, resulted in a lower mean nodule number (2.5, SE = 0.5) than did the medium- or low-frequency irrigation (3.9, SE = 0.63).

In Expts. 1, 2, and 3, 'Royal Gala' and 'Regal Gala' had an overall mean of 2.5 nodules per apple with 68% of apples having one or more nodules. Nodule frequency was 20% in the 'Splendour' sample and 100% in '8S 27-2'.

Discussion

Nodules were detected even in young developing fruit ≈3 cm in diameter. At this stage of development, nodules were pale green and did not strongly contrast in color with the developing apple cortex, which made them difficult to see. At commercial harvest, the nodules in the 'Gala' strains remained green and were not conspicuous, but became more so as the fruit continued to ripen, and bundles changed from pale green to yellow to brown and, sometimes to red. The color of the nodules paralleled this transition. In other cultivars, such as '8S 27-2', the nodules were dark brown or black at commercial harvest maturity and were readily apparent on cutting the fruit in an area that exposed them. In both cultivars, red coloration developed synchronously in both the vascular bundles and the nodules in overmature fruit. The diameter of the nodules was seldom >2 mm and they were not conspicuous in the 'Gala' strains unless the fruit were overmature. Fruit quality of cultivars with dark nodules at maturity was reduced, since brown or dark color in apple cortex is unattractive and is often associated with cell death or breakdown caused by senescence, infection, mineral deficiency, or other causes. The dark coloration, characteristic of the nodules described here, was not a result of cell death or senescence, since the nodule initials were observed in young developing fruit.

The sequential development of corking disorders of apple, such as bitter pit and cork spot, has been described (Simons et al., 1971) and may have relevance to the nodules described here, since corking disorders are often contiguous with vascular tissue. Corking lacunae develop in the outer cortex, usually in the basin portion of the fruit, and their initial development is associated with necrosis of secondary vascular bundles, and with sepal, petal, and style abscission zones that produce cork cambium cells. As the corky spots develop, plasmolysis of cells occurs, resulting in the lacunae. Thickening of the walls of nearby cortical cells often occurs, some of which accumulate starch and initiate cell proliferation. Corky or callus cells around the lacunae characterize the disease. Fruit developing cork spot, in the later stages of fruit development, sometimes have proliferating cells associated with vascular tissue at the core line. This is associated with senescence and necrosis of the vascular tissue and significant tissue breakdown.

The vascular nodules occurred at the shoulder of the apple, in the inner cortex, and did not

appear to be associated with necrosis of vascular tissue, since both nodules and vascular tissue accumulated anthocyanin, a metabolic process, in overmature fruit. Both disorders involve cell proliferation in young fruit well before maturity since nodule initials were present in young fruit. The nodules often developed a central cavity surrounded by lignified cells, while corky spots are characterized by large cavities without associated lignification. The large primary vascular bundles of the core line were integrated with the nodules and characteristic of them.

The nodules associated with vascular bundles were present in all the collections of fruit examined in these experiments, but the frequency was variable, ranging from 0–10 or more per fruit. Genetic differences appeared to influence nodule frequency. '8S 27-2' had a high mean nodule number and large conspicuous nodules. 'Regal Gala', in general, had more nodules than 'Royal Gala', suggesting the possibility of a genetic difference between the strains. Differences in nodule frequency due to environmental factors did occur, since there was considerable variation from tree-to-tree and among fruit from the same tree. Water stress, among other factors, has been reported to influence vascular bundle characteristics in some other crops, and, in these experiments, the water stress from the 21- and 7–14-d irrigation cycles generally increased nodule frequency in 'Royal Gala' and 'Regal Gala' compared with daily watering.

Future studies that describe, in greater detail, the developmental anatomy of the nodules and compare nodule development to other apple fruit disorders that involve cell proliferation near vascular tissue would be useful. Also, studies to investigate genetic and additional environmental factors influencing their development would contribute to a more comprehensive understanding of nodule formation.

Literature Cited

- Cachorro, P., A. Ortiz, A. Ros-Barcelo, and A. Cereda. 1993. Lignin deposition in vascular tissues of *Phaseolus vulgaris* roots in response to salt stress and Ca^{2+} ions. *Phyton* (Horn) 33:33–40.
- Clark, G. 1981. Staining procedures. 5th ed. Williams and Wilkins, Baltimore.
- Garcia-Mas, J., R. Messegue, P. Arus, and P. Puigdomenech. 1996. Accumulation of specific mRNAs during almond fruit development. *Plant Sci.* 113:185–192.
- Hsia, C.L., B.S. Luh, and C.O. Chichester. 1965.

Table 1. Effect of irrigation method and frequency and strain of apple on mean nodule frequency per fruit. Drip irrigation treatments received 16 L/tree, micro sprinkler treatments, 36 L/tree during each irrigation cycle. Standard error in parentheses. N = 20.

Irrigation cycle time (d)	Sprinkler type			
	Drip		Micro	
	Royal Gala	Regal Gala	Royal Gala	Regal Gala
1	0.45 (0.24)	3.6 (0.98)	0.9 (0.54)	5.3 (0.79)
7–14	1.8 (0.43)	6.0 (0.67)	1.45 (0.98)	6.3 (0.66)
21	1.7 (0.42)	5.0 (0.90)	0.75 (0.29)	8.4 (1.45)

Table 2. Effect of weed control and nitrogen application on mean nodule frequency per fruit. Standard error in parentheses. N = 50.

Weed control	Nitrogen (kg·ha ⁻¹)	
	80	200
Complete	1.14 (0.39)	1.16 (0.40)
Minimal	1.10 (0.41)	1.18 (0.40)

- Anthocyanin in freestone peaches. *J. Food Sci.* 30:5–12.
- MacArthur, M. and R.H. Wetmore. 1939. Developmental studies in the apple fruit in the varieties McIntosh Red and Wagener. *J. Pom. Hort. Sci.* 17:218–232.
- Neilsen, G.H., E.J. Hogue, and M. Meheriuk. 1999. Nitrogen fertilization and orchard floor vegetation management affect growth, nutrition and fruit quality of 'Gala' apple. *Can. J. Plant Sci.* 79:379–385.
- Neilsen, G.H., P. Parchomchuk, D. Neilsen, R. Berard, and E.J. Hogue. 1995. Leaf nutrition and soil nutrients are affected by irrigation frequency and method of NP-fertigated 'Gala' apple. *J. Amer. Soc. Hort. Sci.* 120:971–976.
- Parra M. del M., O. del Pozo, R. Luna, J.A. Godoy, J.A. Pintor Toro, and O. del Pozo. 1996. Structure of the dehydrin tas 14 gene of tomato and its developmental and environmental regulation in transgenic tobacco. *Plant Mol. Biol.* 32:453–460.
- Prandl, R., E. Kloske, and F. Schoffl. 1995. Developmental regulation and tissue specific differences of heat shock gene expression in transgenic tobacco and *Arabidopsis* plants. *Plant Mol. Biol.* 28:73–82.
- Ravi, V. and P. Indira. 1996. Anatomical studies of tuberization under water deficit stress and stress free conditions. *J. Root Crops* 22:105–111.
- SAS Institute. 1989. SAS/STAT user's guide, ver. 6, 4th ed. vol 2. SAS Inst., Cary, N.C.
- Saunders, D.C., J.D. Cury, P.M. Deyton, and R.G. Gardner. 1996. Assessing vascularization response of three tomato (*Lycopersicon esculentum* Mill.) cultivars to soil type, nutrient stress and water stress. *HortTechnology* 6:405–408.
- Simons, R.K. 1968. The morphological and anatomical comparison of some physiological disorders in apple. *Proc. Amer. Soc. Hort. Sci.* 93:775–791.
- Simons, R.K., F.N. Hewetson, and M. C-Y. Chu. 1971. Sequential development of the 'York Imperial' apple as related to tissue variances leading to corking disorders. *J. Amer. Soc. Hort. Sci.* 96:247–252.
- Yang, P.-F., G.-M. Ying, L.-L. Xia, L. He, F.-T. Yu, W.-X. Xin, G.-S. Yi, P.-F. Yang, M.-Y. Gao, L.-X. Luo, H. Lui, T.-Y. Fan, X.-X. Wang, S.-Y. Guo, and D.-W. Zhu. 1995. Studies on the cell micro-ultrastructure of the secondary vascular tissue of the stem of the spur-type apple. *Acta Hort.* 403:161–168.
- Zielinski, Q.B. 1955. Modern systematic pomology. W.C. Brown, Dubuque, Iowa.