FRUITS 74(3), 117–123 | ISSN 0248-1294 print, 1625-967X online | https://doi.org/10.17660/th2019/74.3.3 | © ISHS 2019

Original article

Discontinuity of xylem function during maturation associated with quality development and calcium allocation in wax apple (*Syzygium samarangense* Merr. & Perry) fruit

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Summary

Introduction – **Wax apple (*Syzygium samarangense* Merr. & Perry) is an economically important fruit crop in tropical Asia. The developmental changes of wax apple fruit, calcium (Ca) accumulation and allocation, and xylem functionality within the fruit were assessed in this study.** *Materials and methods* – **Fruits were sampled at eight developmental stages and subjected to analyses for quality associated properties. Functionality of xylem inside fruit was evaluated with a methyl blue dye-infusion approach and the spatial distribution of Ca in mature fruit was assessed.** *Results and discussion* – **The growth of a wax apple fruit expressed a typical single sigmoidal curve against time. The breaker stage or the beginning of skin coloration signaled the onset of maturation process as indicated by a rapid increase in total soluble solids and skin anthocyanin content, and a simultaneous decrease in titratable acidity. Xylem remained fully functioning through the whole fruit before the breaker stage. However, a sharp functional decline in xylem toward the distal end of the fruit was observed after the breaker stage. Total Ca content continued to increase but overall Ca concentration reduced after the breaker stage. At harvest, a mature fruit expressed a high to low Ca conc. gradient from the proximal to the distal end.** *Conclusion* – **The low Ca conc. in the flesh near the distal end could be attributed to the loss of xylem function after the breaker stage. Therefore, early and efficient Ca uptake is essential for maintaining high Ca conc. in the distal end where corky calyx end disorder, a speculative Ca deficiency symptom of wax apple fruit, may occur.**

Keywords
Taiwan, wax apple, *Syzygium samarangense*, apoplastic dye, calcium deficiency, corky calyx end disorder, veraison

Significance of this study

What is already known on this subject?

- The corky calyx end (CCE) disorder, a speculative Ca deficiency problem, develops symptoms in the flesh near the distal end of mature wax apple fruit.

What are the new findings?

- Total Ca content continuously increased through the developmental period but average Ca conc. dramatically declined after the breaker stage. Meanwhile, loss of xylem function at the distal end occurred. At harvest, the lowest Ca conc. was detected in the flesh near the calyx end.

What is the expected impact on horticulture?

- Orchard practices that encourage Ca uptake and translocation during the early fruit developmental stage when xylem is fully functioning are critical for maintaining high Ca conc. and reducing the potential risk of CCE disorder in wax apple fruit production.

Introduction

Wax apple (*Syzygium samarangense* Merr. & Perry), an economically important fruit crop in tropical Asia (Paull and Duarte, 2012), is especially prized in Taiwan (Shü *et al.*, 2007). The production of premium wax apple fruit can only be achieved by intensive labor and technical inputs. However, the orchard task largely relied on growers' experience and often challenged by the fluctuating climate and soil conditions. The corky calyx end (CCE) disorder that expresses skin discoloration and corky flesh texture near the calyx (the distal end) of a mature wax apple fruit is a physiological malfunction which commonly occurs in orchards where intensive fertilization and irrigation managements were practiced (Chen, personal observation). The CCE disorder has been speculated to be the result of calcium (Ca) deficiency (Lin and Tsai, 2004) and the overall Ca concentration was often low in a mature fruit that expresses CCE symptom (Chen *et al.*, 2014). We also found that early post bloom Ca sprays on young fruits mitigated the incidence of CCE disorder (Chen *et al.*, 2014). However, little scientific evidence on the developmental physiology of wax apple fruit is available to yield a fundamental understanding of CCE disorder.

Calcium is a mineral element essential for maintaining the stability and integrity of cell membrane and cell wall as well as playing key roles in many physiological activities (Hocking *et al.*, 2016). Ca is transported into a fruit through xylem during early developmental stages (Cabanne and Donèche, 2003; Mengel and Kirkby, 2001). In many mature fruits, Ca conc. is highest in the peel and lowest in the flesh adjacent to the peel, and a conc. gradient from high to low

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has been reported in the proximal-distal direction (Adams and Ho, 1993; Ferguson *et al.*, 1999). Several important fruit physiological disorders, e.g., the bitter pit in apple (*Malus ×domestica* Borkh.) and the blossom end rot (BER) in tomato (*Lycopersicon esculentum* Mill.), have been attributed to Ca deficiency (Djangsou *et al.*, 2019) and the symptom often appears in locations having the lowest Ca conc. (Adams and Ho, 1993; Perring and Pearson, 1986). The visual symptom of wax apple CCE disorder is similar to that of bitter pit in apple and BER in tomato.

The objectives of this study were 1) to document the developmental physiology of wax apple fruits, 2) to quantify the conc. and allocation of Ca in a mature wax apple fruit, and 3) to inspect the functionality of xylems in developing wax apple fruits. Previous studies found that the xylem sap inflow in grape (Greenspan *et al.*, 1994) and kiwifruit (Dichio *et al.*, 2003) berries ceased upon the onset of fruit maturation. This phenomenon might result in the temporal change in accumulation or spatial distribution of Ca in mature wax apple fruits as well. We therefore hypothesized that a similar xylem functionality loss occurs in wax apple fruits, leading to a low Ca conc. toward the calyx end that is prone to CCE disorder.

Materials and methods

Experiment one:
Changes in quality-associated properties, xylem functionality, and Ca distribution during fruit development

Plant materials

Nine-year-old 'Big Fruit' wax apple trees in the experimental orchard of Kaohsiung District Agricultural Research and Extension Station (120.5E; 22.7N, 35 m alt.) in Pingtung, Taiwan were used for sampling. Trees were 3 m in height and spaced at 7 × 6 m. Commercial pruning, fertilization and pest control commonly used in this region were practiced. After full bloom on 11 April 2014, fruit clusters on trees with similar vigor and yield were hand thinned to approx. 200 clusters per tree and five to six fruits per cluster. Fruit clusters were enclosed in paper bags 21 days after full bloom (DAFB). Fruit bagging is a common practice in wax apple cultivation for fruit protection and quality improvement (Shü *et al.*, 2007). The bags were not removed until the commercial harvest maturity at 59 DAFB.

Fruit clusters from the middle section of the canopy were randomly sampled by hand at eight developmental stages defined by Shu *et al.* (1998) with slight modification: young fruit I and II (corresponding to 3 and 13 DAFB, respectively, for the summer fruiting cycle), small bell (23 DAFB), big bell (28 DAFB), breaker (34 DAFB), small red (38 DAFB), big red (45 DAFB) and harvest (59 DAFB) (Figure 1). On each sampling date, fruits from twelve clusters were collected and analyzed immediately.

Quality-associated properties

The fresh weight (FW), dry weight (DW), longitudinal length and maximum diameter of 12 fruits, one from each cluster sample, were measured. The relative growth rate (RGR) was calculated according to Hunt *et al.* (1992):

$$RGR = (W1-W0)/W0/(d1-d0)$$

where W1 and W0 were the FW registered at the sampling date (d1 DAFB) and at the previous sampling date (d0 DAFB), respectively.

DW of the sample was determined after oven drying at 65 °C for 7 d. Water content was calculated by:

$$(FW-DW)/FW \times 100\%.$$

After the big bell stage (28 DAFB), total soluble solids content (TSSC) and titratable acidity (TA) of 12 fruits, one from each cluster sample, were measured. TSSC was measured using a refractometer (PAL-1, ATAGO, Tokyo, Japan) and TA of the juice was determined at an end point of pH 8.1 using a titrator (TA-70, TOA Electronics Ltd., Tokyo, Japan).

Anthocyanin conc. of the fruit skin was determined after the big bell stage according to the method described by Shü *et al.* (2001). On each measuring date, six fruits were used and five peel discs (2.77 cm^2 in total) were collected from the widest part of each fruit. The discs were soaked in 3 mL 1% HCl-methanol overnight in the dark at 4 °C. The absorbance of the extraction at 530 nm wavelength was measured with a spectrophotometer (U-2800A, Hitachi, Tokyo, Japan).

Xylem functionality

Xylem functionality was examined using a dye-infusion method modified from Dražeta *et al.* (2004). Six fruit samples with a piece of 2-cm long pedicel attached to each fruit

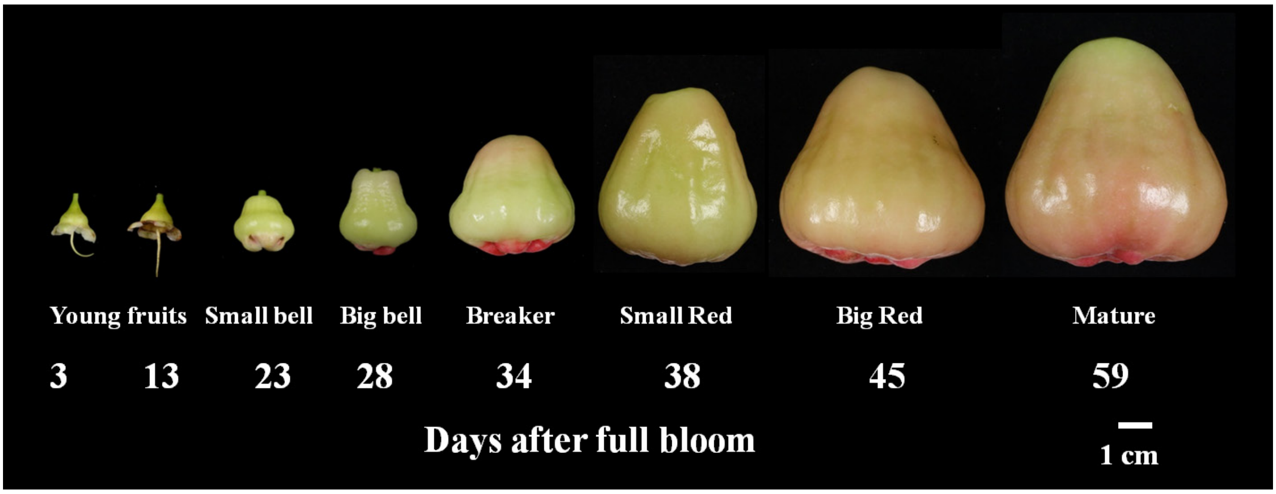

Figure 1. Developmental stages of 'Big Fruit' wax apple fruits as modified from Shu *et al.* (1998). The number below each stage indicates days after full bloom of the 2014 summer cropping cycle in Pingtung, Taiwan.

were examined at each developmental stage. The fruit sample was placed upside down and the pedicel was immersed in a 15-mL test tube filled with 1% (w/w) aqueous methyl blue, a visually distinctive water soluble dye that is readily absorbed and transported through xylem, for 24 h. Two 2-mm thick transverse sections, one from the widest part (the distal end) of the fruit and the other from the proximal end approx. 5 mm beneath the pedicel, were sampled from each fruit. The number and percentage of vascular bundles expressing blue color in each section were calculated to indicate the functionality of xylem.

Temporal changes in calcium conc.

Six fruit samples at each developmental stage were weighed, rinsed in deionized water, diced into approx. 2-cm^3 cubes and then frozen at -80 °C until analysis. The frozen samples were vacuum dried at -20 °C for 1.5 h, followed by -10 °C for 2 h, -5 °C for 2.5 h, 4 °C for 3 h, 12 °C for 5 h, 18 °C for 5 h and finally 22 °C for 5 h with a custom-assembled vacuum dryer (Minguan Instrument Co., Taichung, Taiwan). The dried sample was ashed at 200 °C for 2 h, followed by 400 °C for 1 h, and finally 550 °C for 1 h. Ca concentration of the ash was determined using an atomic absorption spectrophotometer (Z-2300, Hitachi, Tokyo, Japan) (Martin-Prevel *et al.*, 1987). Total Ca content per fruit and calcium conc. based on dry weight (mg kg^{-1} DW) was calculated.

Experiment two:
Spatial distribution of calcium in mature fruits

Plant materials

Fruits from a commercial orchard in Chungjhih (120.6E; 22.7N, 66 m alt.), Pingtung, were used in this study. Trees were 7-year-old, 2.5 m in height and spaced at 6 × 6 m. Commercial pruning, fertilization and pest control commonly used in this region were practiced. After the bloom on 5 Nov. 2013, trees with similar vigor and yield were hand-thinned to approx. 120 clusters per tree and six fruit per cluster. Mature fruits were sampled on 22 Jan. 2014 for analysis.

Calcium analysis

Four samples of eight fruits each were randomly harvested by hand from branches in the middle section of the canopy. Fruits were rinsed in deionized water and wiped dry. Each fruit was dissected into nine parts (Figure 2) and each individual part from the eight fruits in a sample was pooled, and then subjected to the preparation and Ca analysis, as described in Experiment one.

Statistical analysis

Changes in fruit FW and DW over time were analyzed with a sigmoidal regression. Xylem functionality and Ca conc. after 28 DAFB were expressed with an exponential decay function. Both regression analyses were conducted using SigmaPlot (version 8.0, SPSS Inc., Chicago, IL, USA). Spatial distribution of Ca within a mature fruit was analyzed by least significant difference (LSD) at $P<0.05$ (Costat, Cohort Software, Monterey, CA, USA).

Results

Changes in the quality-associated properties, xylem function, Ca content and concentration

The fresh weight (FW) and dry weight (DW) of a developing 'Big Fruit' wax apple expressed a single sigmoidal pattern against time ($R^2 = 0.96$ and 0.97, respectively) (Figure 3A-B). A maximal RGR of 0.437 g g^{-1} day^{-1} was recorded at the big bell stage around 29 DAFB, followed by a decline to 0.123 g g^{-1} day^{-1} after the breaker stage (Figure 3A). Water content increased to 92% by 34 DAFB during the breaker stage, then slightly decreased to 88% by harvest (59 DAFB) (Figure 3B). The length and maximum diameter followed a pattern

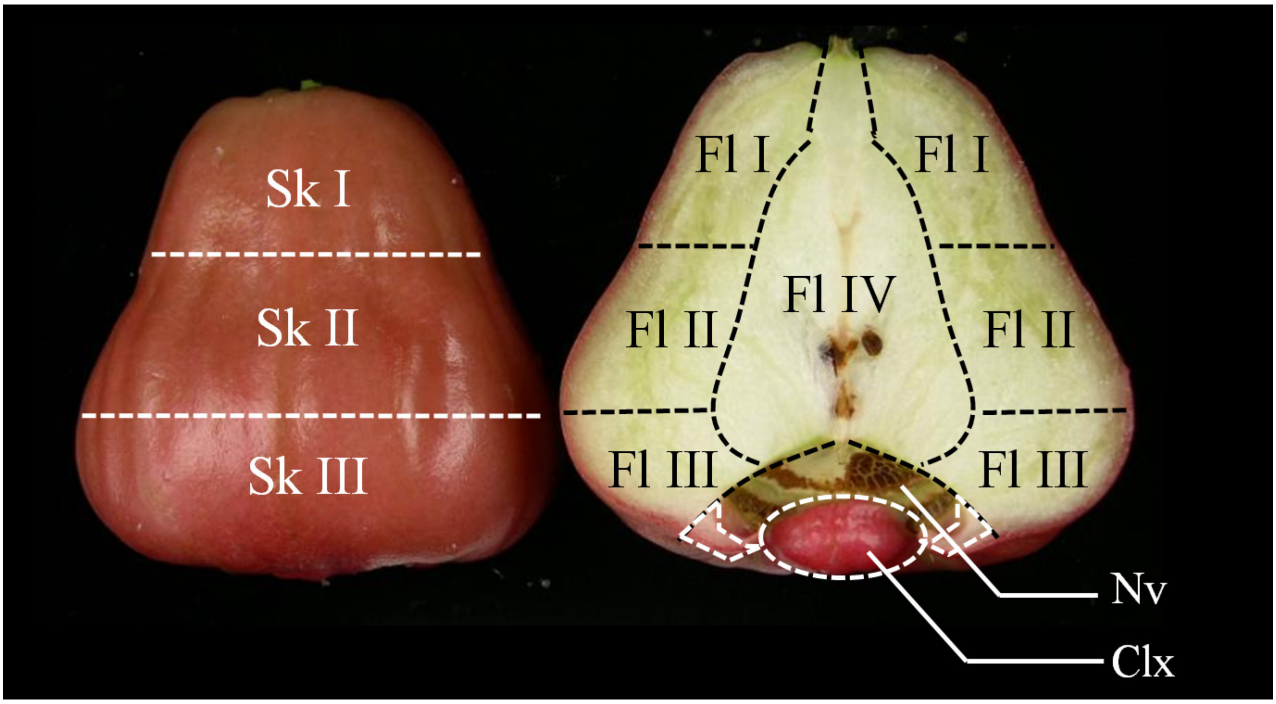

FIGURE 2. Sections of mature 'Big Fruit' wax apple fruit for calcium analysis. Sk I, fruit skin around the proximal end; Sk II, fruit skin from the middle section of the fruit; Sk III, fruit skin around the distal end; Fl I, flesh near the proximal end; Fl II, flesh from the middle section of the fruit; Fl III, flesh near the distal end; Fl IV, the internal spongy-like flesh, placenta, and the flesh adjacent to the pedicel; Nv, the skin tissue inside the navel; Clx, the calyx lobes.

similar to FW and DW against time (Figure 3C).

Sweetness as indicated by TSSC was low at the beginning of the breaker stage followed by a significant and consistent increase corresponding to the decrease in TA and water content (Figure 4A). By harvest, TSSC and TA of a mature 'Big Fruit' wax apple were about 11 °Brix and 0.2%, respectively.

Coloration of the fruit skin other than the calyx lobes began at 34 DAFB as indicated by an increase in the absorbance value of skin anthocyanin extract (Figure 4B). Pigmentation first appeared around the proximal end, then gradually progressed toward the distal end (Figure 1). By harvest, an increase >10-fold in absorbance value ($OD_{530\ mm}$ = 0.251) was recorded, as compared with the breaker stage ($OD_{530\ mm}$ = 0.021) (Figure 4B).

Before the big bell stage (28 DAFB), xylems in fruits after infusion with methyl blue were all fully stained and visually distinguishable in flesh near the distal end of the fruit (Figure 5A). The loss of xylem function in this region as estimated by the percent absence of blue stains (Figure 5B) expressed an exponential decay against time ($R^2 = 0.93$) after 28 DAFB (Figure 4B), corresponding to the increase in TSSC and coloration of the fruit skin. No stained xylem was observed at the distal end after 45 DAFB (Figure 5C), while most xylem bundles near the proximal end still remained functioning as indicated by the presence of blue color (Figure 5D).

Relatively high Ca concentrations were measured in young 'Big Fruit' wax apple before the big bell stage. A sharp decrease in Ca conc. occurred between the big bell and the breaker stage, followed by a gradual but consistent decline until harvest (Figure 4C). Regardless, the total Ca content of whole fruit increased consistently through the fruit development period.

Spatial distribution of calcium concentration in mature fruits

Calcium concentrations in a mature wax apple fruit were characterized among different parts (Table 1). The proximal skin (Sk I) had the highest Ca conc. while the distal flesh

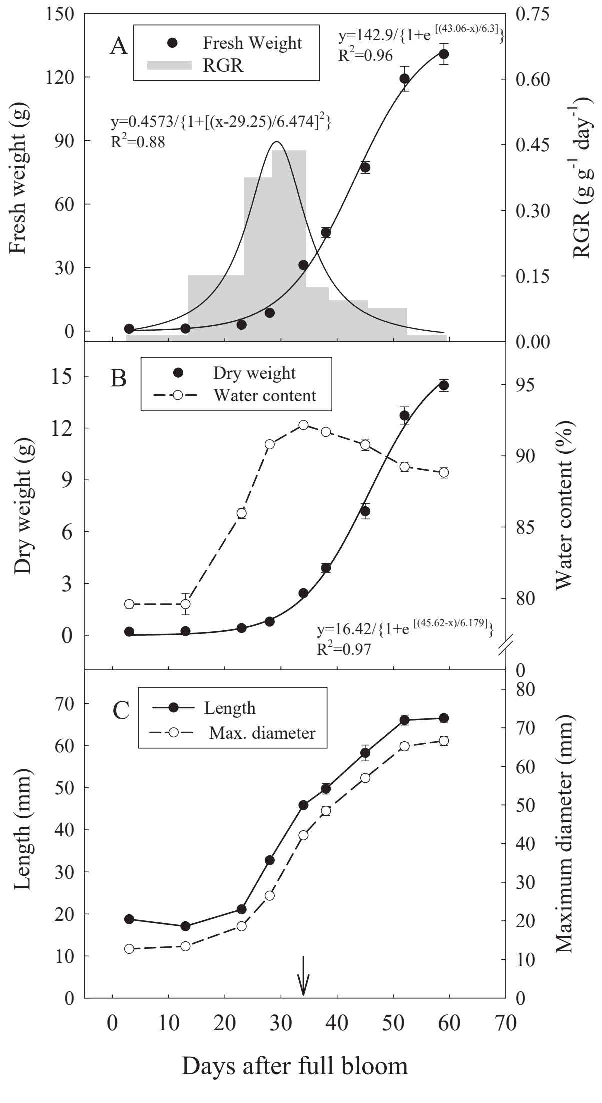

Figure 3. Changes in A) fresh weight and relative growth rate, B) dry weight and water content, and C) length and diameter in developing 'Big Fruit' wax apple fruits. Vertical bars represent standard error (*n* = 12).

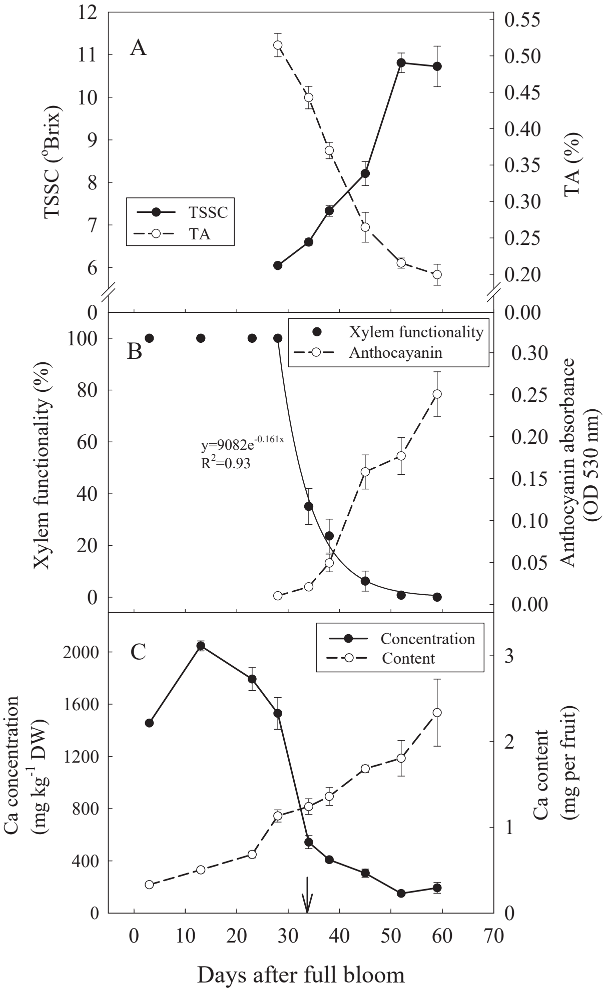

Figure 4. Changes in A) total soluble solids content (TSSC) and titratable acidity (TA), B) functionality of xylem in the distal end and whole-fruit anthocyanin concentration (expressed as O.D. at 530 nm), and C) calcium concentration and content in developing 'Big Fruit' wax apple fruits. Vertical bars represent standard error (*n* = 12 for TSSC and TA; *n* = 6 for the rest). The arrow indicates the breaker stage at 34 DAFB.

TABLE 1. Spatial distribution of calcium concentration within mature ‘Big Fruit’ wax apple fruit.

Code[z]	Sample	Position	Ca conc. (mg kg^{-1} DW)
Sk I	Skin	Proximal	1263 a[y]
Sk II	Skin	Middle	611 c
Sk III	Skin	Distal	459 de
Fl I	Flesh	Proximal	359 e
Fl II	Flesh	Middle	201 f
Fl III	Flesh	Distal	124 f
Fl IV		Core	359 e
Nv		Navel skin	778 b
Clx		Calyx lobe	519 cd

[z] Fruits were dissected and coded as described in Figure 2.

[y] Means with the same letter were not significantly different at $P<0.05$ by LSD test ($n=4$).

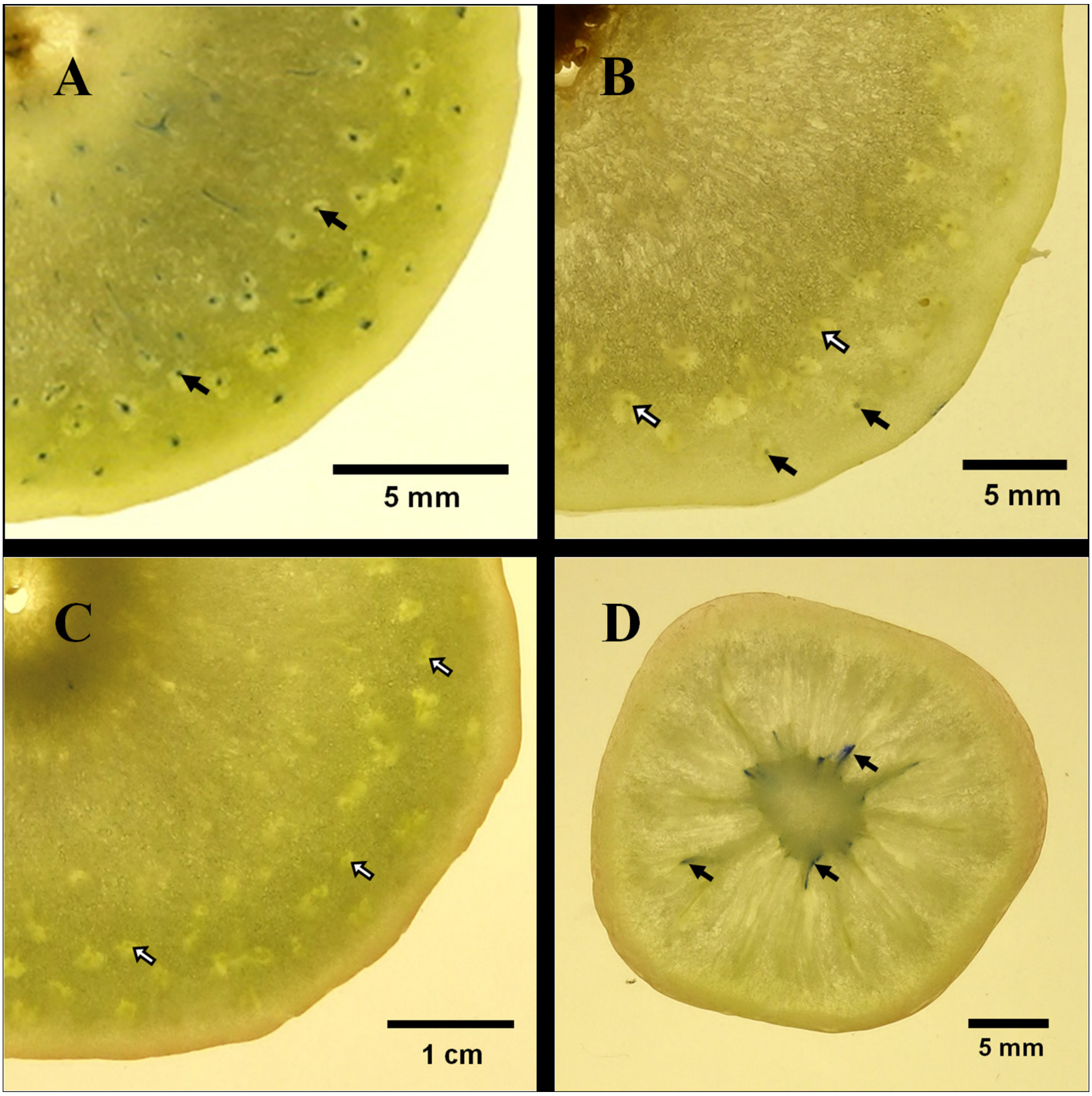

FIGURE 5. Transverse sections of the distal end at A) 28, B) 34 and C) 45 DAFB, and of the pedicel end at D) 45 DAFB of ‘Big Fruit’ wax apple fruits infused with methyl blue. Functioning xylem as indicated by the presence of blue stains (closed arrows) are discriminated from dysfunction xylem as indicated by the absence of blue color (open arrows).

(Fl III) had the lowest. Overall, a decline in Ca conc. from the proximal end (pedicel) toward the distal end (calyx) was observed both in the skin and in the flesh of a mature 'Big Fruit' wax apple.

Discussion

The growth of a 'Big Fruit' wax apple expressed a typical single sigmoidal curve against time as reported previously in other cultivars (Khandaker and Boyce, 2016; Shu *et al.*, 1998). In this study, the initial of rapid growth and the highest relative growth rate were observed between the big bell and the breaker stage (Figure 3A). Concurrently, noticeable developmental transitions in many quality associated properties, *e.g.*, TSSC, TA, coloration and Ca conc., as well as a dramatic decline in xylem physiological functionality, also occurred during this period, indicating that the breaker stage, similar to the veraison in grape, signals the onset of maturation in wax apple.

In grape, changes upon veraison have been comprehensively documented and serve as a model for fruit developmental physiology. Correlations between accumulations of soluble solids and anthocyanin (Casellarin *et al.*, 2011), decline in sap flow, increase in xylem hydraulic resistance (Choat *et al.*, 2009; Greenspan *et al.*, 1994; Tyerman *et al.*, 2004) and osmotic potential during post-veraison maturation process (Bondada and Keller, 2012; Wada *et al.*, 2008) have been reported. Based on these findings, mechanisms to integrate the water status and fruit maturation have been proposed. Previous studies in wax apple (Wang, 2001) reported a positive correlation between anthocyanin accumulation and TSSC during fruit maturation similar to those reported in grape (Chou and Li, 2014). The present study is the first report in wax apple to validate the concomitant changes in xylem functionality and quality associated properties during fruit development.

The decline in xylem function upon the onset of maturation has been observed in other fruit crops and various mechanisms have been proposed. In grape berries, the high hydraulic resistance in xylem after veraison was attributed to the high viscosity in apoplast caused by solutes accumulation (Choat *et al.*, 2009). The reduction in xylem conductance in a developing apple was attributed to the physical disruption of the xylem bundles due to flesh cell expansion (Dražeta *et al.*, 2004). A decline in xylem flow into a mango fruit prior full maturity was attributed to embolism in the pedicel (Nordey *et al.*, 2015). Our results showed that vascular bundles in the proximal end of a wax apple fruit remained functioning at 45 DAFB (Figure 5D), suggesting physical integrity of the xylem still maintained inside the pedicel prior harvest, and the discontinuity of xylem function occurred inside the fruit beginning at the distal end.

Timing and efficiency of Ca uptake into a fruit varies among fruit species but the most efficient Ca uptake usually occurs in the early fruit developmental stage. For example, 80% of Ca in a ripe melon (*Cucumis melo* L.) fruit was transported into the fruit within 20 days post-anthesis (Bernadac *et al.*, 1996). Cabanne and Donèche (2003) reported that the Ca content in developing grape berries increased sharply before veraison and then remained relatively consistent until harvest. However, Rogiers *et al.* (2000) reported a linear increase in Ca content throughout the fruit enlargement and ripening phase. Our results showed that although overall Ca conc. declined, the discontinuity of xylem function inside a wax apple fruit after the beginning of ripening process did not stop Ca uptake into the fruit as evidenced by the continuous increase in total Ca content (Figure 4C) but might have decelerated Ca allocation to the distal end (Table 1). Ca accumulation usually mirrors the capacity of xylem flow (Rogiers *et al.*, 2000), suggesting that the low Ca conc. in the distal end of a mature wax apple fruit might be a result of the restriction of xylem sap flow inside the fruit after the onset of maturation process. The spatial distribution pattern of Ca conc. in wax apple fruit is similar to that in tomato (Adams and Ho, 1993) and apple (Ferguson *et al.*, 1999).

A wax apple fruit is botanically a pseudocarp with fleshy receptacle enclosing a pair of spongy and often parthenocarpic carpels (Figure 2). The vascular bundles in a wax apple fruit consists of two distinctive systems, the carpellary and the cortical system (Chen, unpublished). The arrangement of the latter appears similar to that in other pome fruits such as apple (Dražeta *et al.*, 2004; Herremans *et al.*, 2015). Apple cultivars susceptible to bitter pit (BP) disorder incurred large or earlier loss in xylem functionality mainly in the primary cortical vascular bundles and a consequent lower Ca conc. as compared to cultivars less susceptible to BP (Dražeta *et al.*, 2004; Miqueloto *et al.*, 2014). The overall low Ca conc. of the flesh (cortex) and the lowest Ca conc. observed in mature wax apple fruit section Fl III (Table 1; Figure 2) also indicates the importance of Ca allocation to this region before the discontinuity of xylem function in the primary cortical vascular bundles. However, the result derived from the present approach does not truly reflect the complicated 3D vasculature wiring network in a fruit and thus the detailed Ca transportation pathway within a developing fruit. The X-ray micro-tomography approach provides a good example of 3D image construction of cortical vascular networks in an apple fruit (Herremans *et al.*, 2015) and may offer a powerful tool to gain insight into this intricate situation in the future.

Conclusions

In field observations, the symptom of CCE disorder always appears first in the flesh near the distal end, corresponding to the lowest Ca conc. status of this region (Table 1). Our hypothesis was supported by the concomitant change between quality associated properties, especially Ca, and the xylem functionality in a developing wax apple fruit. The result also indicates the importance of Ca uptake and allocation in early fruit developing phases. Practical strategies, such as post-bloom Ca spray to secure efficient Ca acquisition and allocation in a young developing fruit to mitigate the potential risk of CCE disorder is recommended. Nevertheless, interactions among the uptake/transport of Ca, canopy and environmental factors and soil management, should be further clarified for better control.

Acknowledgments

We thank Dr. Ching-Lung Lee and Dr. Jer-Chia Chang for their comments on this work. We also thank Mr. Meng-Hsun He and Yi-Ying Li for their assistances in measurements. The study was supported by the Council of Agriculture, Taiwan (Project ID: 104AS-9.4.3-KS-K1).

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Received: Oct. 10, 2018
Accepted: Apr. 3, 2019