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Postharvest internal browning of pineapple fruit originates at the phloem

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ABSTRACT

A typical symptom of postharvest chilling injury (PCI) in pineapple fruit (*Ananas comosus* (L.) Merr.) is internal browning (IB) near the fruit core. Since vascular bundles (VBs) are localized to this region, it was hypothesized that the VBs might be the site of IB. To test this, the anatomy and histochemistry of VBs during chilling stress in four pineapple cultivars with different levels of sensitivity to PCI were examined. Fruit were stored at 10 °C for up to three weeks to stimulate translucency symptoms (TS; the initiation of IB). After three weeks of chilling exposure, the cultivars 'MD2' showed 0%, 'Pattavia' and 'Savee' showed 10–16%, and 'Trad Sri Thong' showed 100% TS and IB symptom. Scanning electron microscopy and in situ histochemical staining techniques that detect enzymes and substrates commonly associated with IB initiation were used in parallel. The TS of pineapple fruit coincided with the collapse of the phloem tissue. The VBs in the tissue where IB was initiated (i.e., the flesh adjacent to the core or F/C) had the highest activity of polyphenol oxidase, hydrogen peroxide, and phenolic compounds. The IB-resistant 'MD2' genotype had fewer VBs, but a greater proportion of sclerenchyma fibers ($P < 0.05$) than did the susceptible 'Trad Sri Thong'. Based on these data, the first report of pineapple IB occurrence in the phloem was proposed.

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1. Introduction

The pineapple (*Ananas comosus* (L.) Merr.) is the 3rd most important fruit traded globally, but it is susceptible to chilling injury (Paull and Lobo, 2012). Chilling injury is a complex physiological disorder that occurs in tropical and sub-tropical fruit, including pineapple, after exposure to low temperatures (0–20 °C) (Paull, 1990; Hassan et al., 2011). Chilling may occur in the field in winter grown fruit causing pre-harvest chilling injury, or after harvest when fruit are stored at low temperatures, i.e., postharvest chilling injury; PCI (Hassan et al., 2011). Losses of up to 80% have been attributed to PCI of pineapples after storage at 10 °C (Wilson Wijeratnam et al., 1993), which reduces wider marketability of the fruit (Stewart et al., 2001). Because of the magnitude of the problem presented by PCI, the focus of this study was limited to this phenomenon and did not include pre-harvest chilling injury.

The severity of PCI depends on the temperature to which fruit is exposed and the duration of exposure. PCI symptoms in pineapple are initiated after 1–3 weeks storage at temperatures below 12 °C followed by a transfer to room temperature (Teisson, 1979). Symptoms mainly occur in the fleshy region adjacent to the core (F/C) (Fig. 1) and the earliest manifestation is tissue translucency symptoms (TS) (Paull and Rohrbach, 1985). As the symptoms intensify, browning or blackening of the tissue becomes noticeable. The resulting fruit damage, also called "internal browning" (IB), "black heart," or "endogenous brown spot," occurs internally with no external signs of injury (Stewart et al., 2002).

The severity of IB symptoms and spatio-temporal development vary by cultivar (Paull and Rohrbach, 1985). IB-susceptible pineapple cultivars belonging to the 'Queen' group show symptoms within 1–2 weeks after storage at temperatures below 10–12 °C (Stewart et al., 2002; Nukulthornprakit and Siriphanich, 2005). The tissue first turns translucent, then brown, in both the core (C) and the fleshy tissue (F) of the fruit (Fig. 1) (Paull and Rohrbach, 1985). IB-tolerant cultivars belonging to the 'Smooth Cayenne' group develop symptoms at a slower rate than do the susceptible types, and the browning is primarily seen in the F region. Pineapples classified as IB-resistant show no TS or IB symptom but may display damage externally (Stewart et al., 2002), also called "common chilling injury" (Hassan et al., 2011).
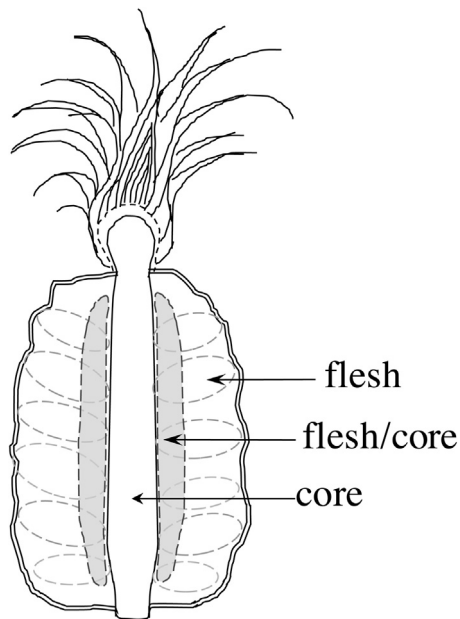
Fig. 1. Schematic representation of a longitudinal section of a pineapple fruit showing the regions observed in this study. The "core" is the central region of the fruit which connects to the stem of pineapple plant. The "flesh" is the edible portion formed by the peduncle, perianth, and bracts. The "flesh adjacent to the core" (flesh/core) is the region where translucency and internal browning symptoms are initiated. In this manuscript, these have been abbreviated to 'F', 'C', and 'F/C', respectively.

IB in pineapple is a result of the metabolic dysfunction from chilling-induced membrane damage (Teisson, 1979; Stewart et al., 2001; Zhou et al., 2003a,b; Nukulthornprakit and Siriphanich, 2005). Loss of organellar structure permits the normally plastidic polyphenol oxidase (PPO) access to phenolic compounds that are usually sequestered to the vacuole (Paull and Rohrbach, 1985; Tomás-Barberán and Espin, 2001; Raimbault et al., 2011). The o-quinones intermediates produced by PPO act on the phenolic compounds, and are then converted to the polymers responsible for the internal browning associated with PCI (Graham and Patterson, 1982; Stewart et al., 2001; Tomás-Barberán and Espin, 2001; Hu et al., 2012). Chilling injury in pineapple is also associated with the production of hydrogen peroxide (H_2O_2) (Nukulthornprakit and Siriphanich, 2005), which ages the cells (Shewfelt and Purvis, 1995; Mittler, 2002). Therefore, higher PPO, phenolics, and H_2O_2 production are common biochemical markers of IB initiation (Tomás-Barberán and Espin, 2001). Although these metabolic changes have been well documented, our current understanding of chilling injury of pineapple is incomplete. This may be due in part to the lack of knowledge regarding the precise site of IB initiation in pineapple fruit.

IB may occur at fruit vascular bundles (VBs) in pineapple. This is plausible even though there are few existing data concerning pineapple fruit anatomy in relation to IB (Teisson, 1977). However, data from tomato (Moline, 1976), mango (Phakawatmongkol et al., 2004), feijoa (Duarte and Paull, 2015), and especially banana (Murata, 1969; Morrelli et al., 2003; USDA, 2004) and avocado (Swarts, 1984; Woolf, 1997; Woolf et al., 2005) support a relationship between VBs and IB. In avocado, fruit VBs separate and become stringy after seven weeks storage at 5.5 °C (Woolf et al., 2005) or four weeks storage at 0.5 °C (Woolf, 1997). The exudate leached from the chilling-damaged VB strands was presumed to cause tissue browning (Woolf, 1997). In banana, after storage at 6 °C for 3 days, chilling-induced damage to fruit peel VBs was detected by histological staining (Murata, 1969). The ruptured VBs were also associated with blackening of the surrounding tissue (Murata,

1969). In pineapple fruit, the F/C region, where IB symptoms are initiated, is the VB-rich region. Thus, investigating the occurrence of the early events of IB development, i.e., TS at the VBs of pineapple fruit, was the aim of this study.

Four pineapple cultivars belonging to two groups, 'Queen' and 'Smooth Cayenne', with three levels of IB sensitivity were used to determine: (1) if IB initiation, i.e., the appearance of translucency symptoms (TS) occurs randomly in any living fruit cell or specifically at the VBs, (2) if the VBs are the sites of biochemical activity associated with TS, and (3) if different IB-sensitive cultivars show differences in VB structure and organization.

2. Materials and methods

2.1. Plant materials

2.1.1. Genotypes

Four pineapple cultivars with different levels of susceptibility to IB were selected for this study: 1) Very susceptible: 'Trad Sri Thong' (TST), 2) Moderately IB-tolerant: 'Savee' (SV) and 'Pattavia' (PTV), and 3) Resistant: 'MD2'. The TST and SV belong to the 'Queen' group of pineapple. These plants were grown in Trat (12°11′35.3″N 102°26′58.5″E) and Chumphon (10°14′19.4″N 99°02′20.1″E) provinces, Thailand, respectively. The PTV and MD2 belong to the 'Smooth Cayenne' group of pineapple and were grown in Rayong (12°55′21.8″N 101°15′08.7″E) province, Thailand.

2.1.2. Planting details

All plantings took place in the rainy season (August 2014) using commercial cultural practices. Plantings were done by a double-row system: with 120 cm spacing between each double-row. Within the double-row, spacing of 60 × 30 cm was made between rows and plants, respectively. N-P-K (16-16-16) and urea (46-0-0) fertilizers were top-dressed one month after planting at the rate of 313 kg/ha, then 14-14-21 was applied 750 kg/ha every three months before maturity. The overall field temperature over the year was 30–35 °C during the day and 22–28 °C at night. After 14 months of planting, pineapple plants were induced to flower by spraying with 50 ppm ethephon and 2% (w/v) urea (approximately 80 mL/plant).

2.1.3. Pineapple fruit used in experiments

The fruit were harvested at the mature green stage, approximately 18 weeks (for TST and SV), or 21 weeks (for PTV and MD2) after floral induction. Fruit were harvested early in the morning and transported to the laboratory within 8 h. On arrival, the fruit were sorted by size and maturity. The uniformity of fruit maturation was determined by visual assessment of the outer exocarp, i.e., the opening stage of the fruitlets or eyes at the stem end of the fruit. Only fruit of which all the fruitlets were totally green were selected for further testing (Selvarajah et al., 2001). At this stage, flesh color was light yellow, and soluble solids content was greater than 11°Brix. Dirt and insects on the fruit were removed using an air blower. All leaves at the stem end were removed and the peduncle was re-cut to remain one inch long. Forty-eight fruit of uniform maturity for each cultivar were selected for chilling injury determinations. Fruit were either examined immediately after harvest (control) or stored at 10 °C and 85% relative humidity (RH) for 7, 14, and 21 days to induce translucency symptoms (TS) or chilling injury (chilled), or stored at 25 °C continuously for the same period (senesced fruit).

2.1.4. Preparation of pineapple tissues

After chilling, six fruit per cultivar were halved longitudinally. The TS and IB degree of each fruit was visually measured (see Section 2.1), and only tissues showing translucency without browning

Table 1

Chilling injury (CI) shown as translucency[a] (TS) or internal browning[b] (IB) symptoms of four pineapple cultivars after storing the fruit at 10 °C for three weeks.

Cultivars	Duration of storage at 10 °C		
	1 wk	2 wks	3 wks
Trad Sri Thong (TST)	0 ± 0	$9 \pm 11b$[a]	$100 \pm 0b$[b]
Savee (SV)	0 ± 0	$0 \pm 0a$	$16 \pm 29a$[a]
Pattavia (PVT)	0 ± 0	$0 \pm 0a$	$10 \pm 10a$[a]
MD2	0 ± 0	$0 \pm 0a$	$0 \pm 0a$
F-test	ns	*	*
% CV	0.0	280.3	135.9

Values are the means of six replicates $\pm$ standard deviation of the mean. Significant differences are indicated by different letters, and were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's test at $P < 0.05$.

[a] Indicates the appearance of translucent spots at the F/C after 10 °C storage. These fruit were used to determine the effect of chilling on the localization of PCI biochemical markers and for fruit vascular bundle examinations (Figs. 2–6).

[b] Indicates the appearance of browning tissue after 10 °C storage. These fruit were not used for any other examinations.

symptoms were selected for further analysis (see Sections 2.2 and 3). Three fruit (representing 3 biological replicates) with a uniform degree of TS were selected from each cultivar for further studies. Tissue samples from three regions: the "core" (C), the "flesh adjacent to the core" (F/C), and the "flesh" (F) regions were taken from the middle of each fruit (Fig. 1). The entire experiment was repeated twice (for PTV and MD2) and three times (for TST and SV) by using fruit from the same field that differed only in the time of flower induction.

2.1.5. Preparation of avocado, banana, and mango tissue

Banana and mango fruit were harvested from orchards in Nakhon Pathom (14°02′07.0″N 99°58′58.2″E) province and taken to the lab within an hour. Avocado fruit was harvested from an orchard in Nakhon Ratchasima (14°37′12.1″N 101°24′51.8″E) province, Thailand, and taken to the lab within 5 h. Uniformly mature green fruit were selected using the guide developed by Kader (2002). Fruit were stored at 10 °C and 85% relative humidity (RH) for 1–4 weeks to induce PCI (Paull, 1990). The pulp, peel, and flesh adjacent to the seed coat of avocado, banana, and mango, respectively, were sampled from three biological replicates (individual fruit) (Paull, 1990). Tissue was fixed as described for pineapple (below), and VBs examined using scanning electron microscopy.

2.2. Examination of chilling injury

2.2.1. Chilling injury (CI) score

Pineapple fruit were halved longitudinally. Translucency symptoms and internal browning due to chilling were visually evaluated and scored based on the percentage of the entire cut surface area of the fruit, showing either translucency or browning, respectively (Teisson, 1979). For example, fruit with 20% of the surface showing translucency or browning was assigned as CI score of 20% TS or 20% IB, respectively.

2.2.2. Localization of biochemical markers for PCI

A thin piece of tissue ($0.5 \times 0.5 \times 0.5$ cm cube) was hand-sectioned transversely with a razor blade perpendicularly to the C at approximately 25–50 μm thick from the indicated regions (Fig. 1; Table 1). All assays were monitored within 4 h after the fruit was cut. The protocols were optimized by varying substrate concentration and incubation times and those that gave the best result were selected for further testing. Controls without staining were performed for each histochemical test (data not shown). Photos from all assays were taken under a stereomicroscope at 10× magnification (Olympus GSWH10X/22 ocular micrometer stereomicroscope; Olympus SZ30, Olympus Optical Co. Ltd., Tokyo,

Japan). Photographs were taken using Dinocapture 2.0 software (Dino-Lite Digital Microscope, Taiwan).

2.2.2.1. Hydrogen peroxide (H_2O_2) production. A thin fruit section was monitored for H_2O_2 by staining with a potassium iodide (KI)/starch reagent. The reagent was composed of 4% (w/v) starch and 0.1 M KI, adjusted to pH 5.0 with potassium hydroxide. The tissue section was flooded with KI/starch reagent for 2–4 h. H_2O_2 production was examined by observing the development of a dark stain on the cut surface (Olson and Varner, 1993).

2.2.2.2. Polyphenol oxidase (PPO) activity. A thin fruit section was sprayed with a 0.04 M aqueous solution of catechol to achieve complete coverage. Regions with polyphenol oxidase activity in the fruit were detected by the development of a brown color over 10 min (Schadel and Walter, 1981).

2.2.2.3. Production of phenolic compounds. The accumulation of phenolic compounds was visualized by drenching the fresh thin sections with 2% (w/v) ferric chloride ($FeCl_3$) for 5–10 min. The tissue was rinsed of excess stain with 95% (v/v) alcohol. A yellow or orange coloration was observed in the presence of phenolic compounds (Reeve, 1951; Schadel and Walter, 1981).

2.3. Examination of fruit vascular bundles (VBs)

2.3.1. Light microscopy

The anatomical structure of fruit VBs was immediately examined after harvest. Fruit was halved longitudinally and a cube of tissue $0.5 \times 0.5 \times 0.5$ cm was hand-sectioned transversely, perpendicular to the C with a razor blade. The sample cubes were fixed following the method described in Ruzin (1999). After embedding in paraffin, the sample blocks were sectioned at a thickness of 10 μm using a microtome (HM 335E, Microtome IMEB Inc., San Marcos, CA). The slides were stained with safranin-O and fast green. In the presence of the stain, lignin, chromatin, and cutin were brilliant red, chloroplasts were pink to deep red, and cellulose and cytoplasm were green. The number, distribution, and changes in VBs and phloem and xylem elements were observed in six fruit as replicates. The sizes of the VBs and xylem elements, the width of the phloem, and the sclerenchyma fiber layer were measured using a light microscope at 40× magnification (Zeiss Axiostar Plus Microscope, Carl Zeiss Microimaging, Inc., Germany). Photographs were taken using Dino-Lite Digital Microscope (Taiwan).

2.3.1.1. Number of VBs. A 20× magnification was used for counting the number of VBs in each region of interest within a radius of 1.5 mm circular field-of-view, using an Olympus GSWH10X/22 ocular micrometer stereomicroscope (Olympus SZ30, Olympus Optical Co. Ltd., Tokyo, Japan).

2.3.1.2. Number of xylem elements. Morphological structures identified as xylem elements were counted from all bundles present in the 0.5×0.5 cm studied section.

2.3.1.3. Size of VB and xylem element. The size of both the VBs and xylem elements was calculated using an ellipse area equation. The size of the VBs was determined from 10–15 bundles per section, and the size of the xylem elements was examined from 30–45 xylem elements per section.

2.3.1.4. Width of phloem and sclerenchyma fiber layer. The width of the phloem and sclerenchyma were determined from 10–15 VBs per section. The width of the phloem layer was determined by measuring the widest distance located between the sclerenchyma and xylem elements as displayed in the image next to the graph (Fig. 3
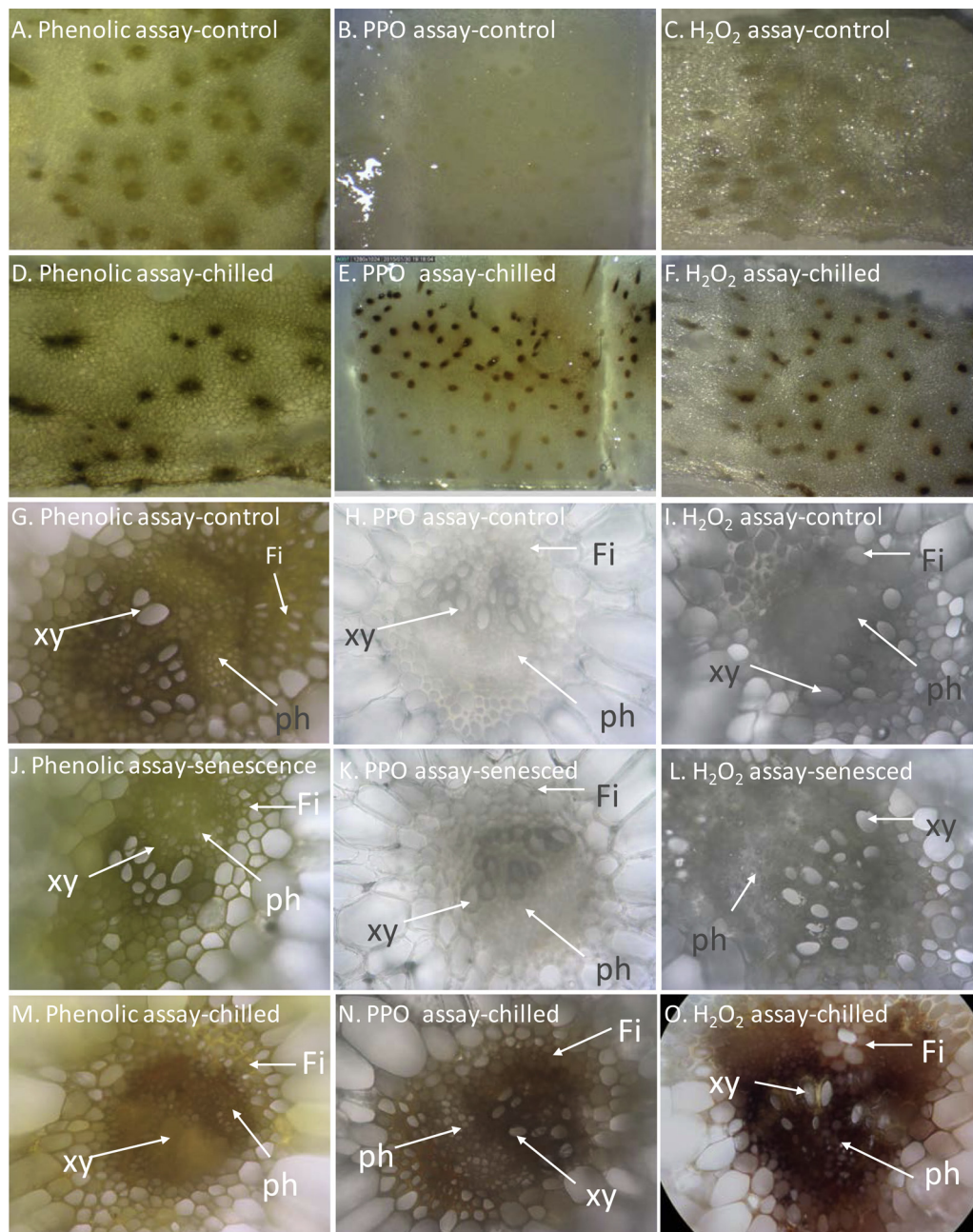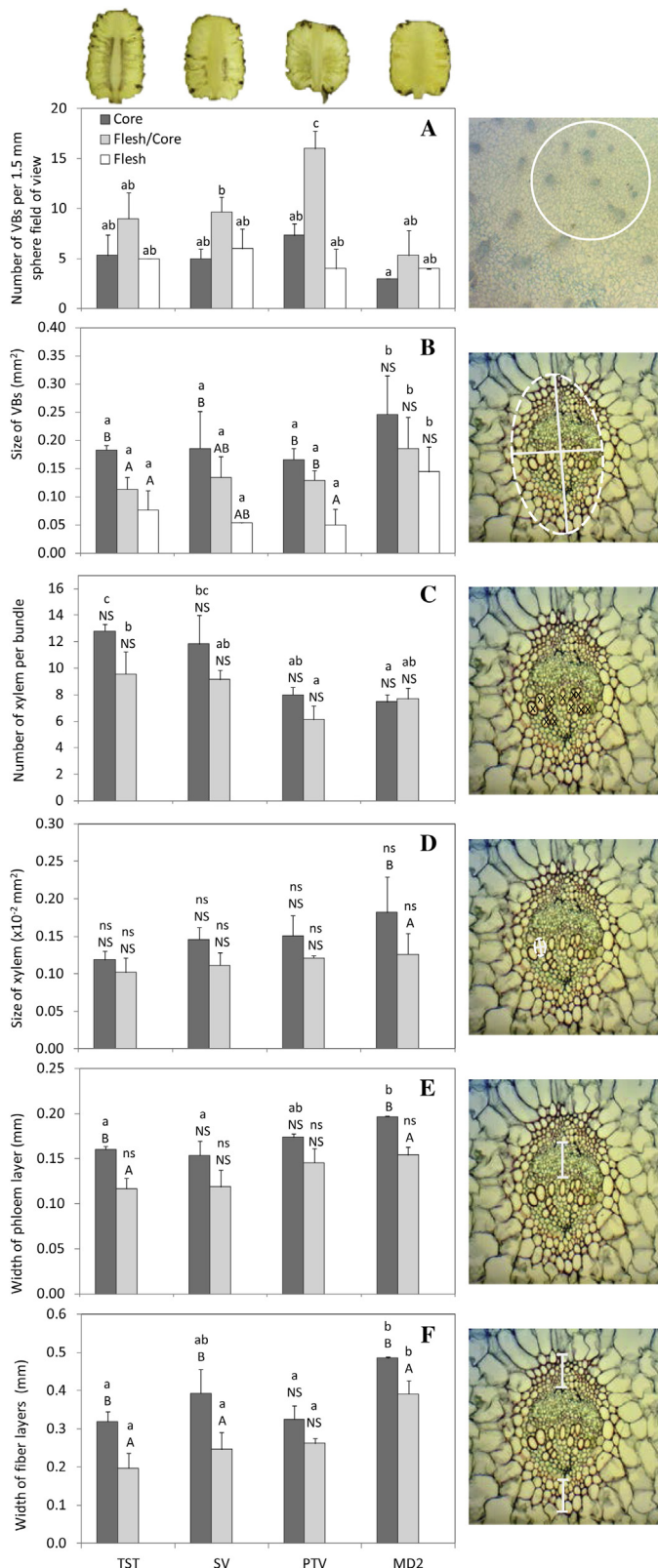
Fig. 2. Histochemical staining assays to determine the localization of phenolic compounds, polyphenol oxidase activity (PPO), and H_2O_2 present in TST pineapple fruit. Control fruit was examined immediately after harvest. The translucent tissue of fruit with no IB symptoms after storage at 10 °C for 14 days was described as 'chilled' tissue. Fruit stored at 25 °C continuously for 14 days was described as "senesced." Photographs were taken from control (A–C and G–I), senesced (J–L) and chilled fruit (D–F and M–O) with a stereo-compound at 10× magnification (A–F) and light compound microscope at 40× magnification (G–O). Tissue was stained with ferric chloride ($FeCl_3$) to indicate the presence of phenolic compounds (A, D, G, J, and M), with catechol to indicate the activity of PPO (B, E, H, K, and N), and with KI/starch to indicate H_2O_2 production (C, F, I, L, and O). The yellow-brown color indicated the presence of phenolic compounds, PPO activity and H_2O_2. Fi = sclerenchyma fiber, ph = phloem, xy = xylem.

E). The width of the sclerenchyma fiber layer was determined by measuring the distance of all sclerenchyma fibers present above and below the xylem elements and phloem tissue as displayed in the image next to the graph (Fig. 3F).

2.3.2. Scanning electron microscopy (SEM)

A small piece of pineapple tissue (approximately $0.5 \times 0.5 \times 0.5$ cm) was cut perpendicular to the C with a razor blade from the translucent tissue. The sample was fixed in a 2.5% (w/v) glutaraldehyde solution containing 0.1 M phosphate buffer pH = 7.0 at room temperature for 72 h. The tissue was rinsed three times for 10 min with the phosphate buffer. The tissue was then rinsed again in distilled water and dehydrated in a series of ethanol solutions in 20% (v/v) increments for 3 steps, starting at 30% (v/v) for 20 min at each step. Before scanning, the pieces were dried to critical-point with liquid CO_2 in a desiccator. The specimens were mounted onto aluminum stubs using a conductive silver glue and sputter coated with gold. SEM was carried out using a JSM-5410LV, JEOL microscope (Tokyo, Japan), at 15–200,000× magnification range, 0.5–30 acceleration voltage range, 6–48 mm work distance, 10^{-12}–10^{-6} A, and 4 nm resolutions.

2.4. Statistical analysis

In this study, a completely randomized experimental design (CRD) with 4 cultivars as treatments was conducted. The CI index was expressed as the mean of six biological replicates. The rest of examinations were averaged from three biological replicates in CRD with 3 regions of the fruit as treatments. Two-way analysis of variance (ANOVA) followed by a Tukey's test at $P < 0.05$ was applied to assess the statistical significance of the differences between the cultivars (SPSS version 19.0; Chicago, IL). In this manuscript, any described differences among means met the $P < 0.05$ threshold.

3. Results

3.1. Examination of chilling injury

3.1.1. Chilling injury scores in different pineapple cultivars

The IB resistant MD2 showed no IB and no TS, and was assigned a score of 0% after being stored at 10 °C for three weeks (Table 1). Still, as reported by Hassan et al. (2011), the peel turned brown after six weeks storage (data not shown). The PTV and SV fruit showed neither TS nor IB after two weeks at 10 °C. However, 10% and 16% TS at the F/C were found in PTV and SV, respectively, after three weeks at 10 °C. The TST was very susceptible as 9% TS were initiated at the F/C after two weeks, and 100% IB developed after three weeks at 10 °C (Table 1).

3.1.2. Phenolic compounds, PPO, and H_2O_2 in chilled TST (IB-susceptible) pineapple fruit

The PCI markers, H_2O_2 and PPO, were not detected by stereomicroscopic analysis of non-chilled fruit (control and senesced fruit) (Fig. 2B and C), whereas the presence of phenolic compounds was observed (Fig. 2A). After the TST pineapple fruit was exposed to 10 °C for two weeks, TS were observed on approximately 9% of the total surface area of the fruit. These spots only appeared in the F/C, not in the C or the F regions. Tissues with these localized translucent areas at the F/C region were carefully sectioned and further investigated. Higher contents of phenolic compounds, PPO activity, and H_2O_2 were detected in the translucent tissue as the development of an intense brown color using our diagnostic histological staining assays (Fig. 2D–F). These areas of TS served as an indicator of IB initiation and were further examined under a light microscopy with higher magnification (40×) (Fig. 2G–O). It was clearly evident that the coloration was more concentrated in the translucent tissue (Fig. 2M–O compared with Fig. 2G–L). Furthermore, PPO activity and H_2O_2 production in the F/C tissue were not detected in non-VB tissue (Fig. 2D–F).

In this paper, we used a non-specific stain as an indicator of PPO activity, which in turn acted as a proxy for IB. Our assay does not allow us to definitely detect PPO activity, but instead, may be a good indicator of the spatio-temporal occurrence of IB. The staining appears only in chilled TST fruit and is absent from tissues with no manifestation of IB, i.e., non-chilled and senescing tissue (Fig. 2H, K, and N). Furthermore, no staining was visible in MD2, a cultivar with no known PPO increase after chilling, and no IB symptoms (Supple-

Fig. 3. Characteristics of the vascular bundles (VBs) in different fruit regions of pineapple genotypes differing in IB susceptibility. Images adjacent to the graph show how the VB characteristics were determined. Three different regions of freshly harvested fruit: the Core, Flesh/Core, and Flesh; from 4 cultivars: TST, SV, PTV, and MD2 were compared. (A) Number of VBs were determined by observing tissue at 20× magnification and counting identified VB structures within a radius = 1.5 mm sphere field-of-view under the stereo microscopy. Different letters were used to differentiate among the means of 3 replicates. (B) and (D) Size of VBs and xylem elements were determined from 10–15 bundles and 30–45 xylem elements per fruit, respectively, and the size was calculated using an ellipse area equation. (C) Number of xylem elements. Morphological structures identified as xylem elements were counted from all bundles present in (A). (E) The width of phloem, and (F) sclerenchyma fiber layers were determined by measuring the distances of all structures identified as phloem and lignified sclerenchyma cells displayed in the picture next to graph. Each data point represents the mean of measurements taken from at least 3 biological replicates, i.e., 3 individual fruit. Vertical error bars represent the standard deviation. On graphs B–F, a capital letter was used to differentiate the means among fruit regions, and small letters were used to determine the difference among cultivars ($P < 0.05$).

mentary Fig. 3; Raimbault et al., 2011). These data indicate that for the purpose of this study, the catechol staining was an adequate marker for IB.

3.2. Vascular bundles (VBs) in relation to chilling injury initiation

The biochemical study suggested that the IB biochemical markers were localized in the VBs in TST. Since IB showed different spatio-temporal development dependent on genotype, VBs in the F, C, and the region connecting the two (F/C) from three other pineapple cultivars were compared to TST (Fig. 3).

3.2.1. Variation in anatomical structure of VB among the flesh, core and flesh/core region

3.2.1.1. Numbers of VBs.
The number of VBs in the F/C region (10–16 bundles per a radius of 1.5 mm circular field-of-view under the stereo microscopy) was double that in the C and the F regions (average 5 and 6 bundles per field-of-view, respectively) in the moderately IB-tolerant PTV. In contrast, the number of VBs was not significantly different in the three fruit regions in TST, SV (5–10 bundles per field-of-view), and MD2 (3–5 bundles per field-of-view) (Fig. 3A).

3.2.1.2. Size of the VBs.
There was some varietal distinction in how this parameter varied across tissues. In IB-occurring cultivars (TST, SV, and PTV), VBs were larger in the C (0.18 mm^2) compared to those in the F/C (0.11–0.13 mm^2), which in turn were larger than those in the F region (0.05–0.07 mm^2). In the moderately IB-tolerant PTV, the VBs sizes in the C and F/C regions were twice as large as those in the F region (0.13–0.16 mm^2 and 0.05 mm^2, respectively). In the IB-susceptible TST, the VB size in the C region was larger than that of the F/C and F. In SV, the size of the VBs in the three regions were not statistically significantly different ($P > 0.05$), but became progressively larger from the F towards the C. However, there was no variation in VB size among the different fruit regions in the IB-resistant MD2 (Fig. 3B). In addition, the VBs in the IB-resistant MD2 were larger in all fruit regions compared with the other three cultivars (Fig. 3B).

It was difficult to accurately measure the size and number of xylem elements, phloem, and sclerenchyma fibers in the F regions because they were small and few in numbers. Therefore, analysis was restricted to the C and F/C regions.

3.2.1.3. Size and number of xylem elements per VB.
The IB-susceptible TST had a higher number of xylem elements per bundle compared to the other cultivars, but there was no difference in xylem element size among cultivars (Fig. 3C and D). Cultivars capable of undergoing IB (TST, SV, and PTV) did not vary either in xylem element size or numbers in the C compared to the F/C. In contrast, the cultivar incapable of undergoing IB had larger xylem elements in the C compared to the F/C region (Fig. 3C and D).

3.2.1.4. Width of the phloem layers.
It was difficult to accurately measure the size and number of phloem because they were small. Therefore, this feature was examined by measuring the width of all phloem tissues (layers) presented within the VB. In addition, the width of these phloem layers were only analyzed from the C and F/C regions since the VBs in the F region were too small to accurately determine the size of the correspondingly small phloem layers. The width of the phloem layers varied among cultivars but did not correlate with IB-symptoms. The phloem layers in the VBs were thicker at the C compared to the F/C region in TST and MD2, the most susceptible and tolerant varieties, respectively. In contrast, phloem layer thickness did not vary among tissues in the IB-moderately tolerant varieties SV and PTV (Fig. 3E).

3.2.1.5. Width of sclerenchyma fiber layers per VB.
The large VB cap consisting of multiple layers of thick cells exterior to the phloem were classified as the sclerenchyma fiber layer, and the widths were determined. In VBs at the C, this layer was larger than that in the F/C region in all cultivars except PTV (Fig. 3F). IB-resistant MD2 was characterized by having sclerenchyma fiber layers that were twice as large, both in the C and F/C regions, compared to the other cultivars. In contrast, there was no difference in size of sclerenchyma fiber layers among the other three cultivars (Fig. 3F).

3.2.2. SEM analysis of VB anatomical structure after translucency induction

3.2.2.1. Spatial analysis of TST pineapple fruit VB.
VB anatomy at the F/C region of control (non-chilled), senesced, and chilled (only those showing TS) tissue of the IB-susceptible TST pineapple fruit were further investigated under a scanning electron microscope (Fig. 4). The VBs consisted of xylem elements, phloem, and VB caps of sclerenchyma fiber. The xylem elements were composed of lignified cells and occupied the center of the VBs. The phloem consisted of thin-walled cells that were small in size and were located adjacent to the xylem elements. A zone of small parenchymal cells surrounding the phloem tissues was modified into sclerenchyma fiber (Fig. 4). Such cells were less abundant or lacking around the VBs, resulting in the small size of the VBs in the F region (Fig. 4L–N).

There were no obvious differences in the non-VB parenchymal cells taken from the F/C region in control compared to chilled fruit (Fig. 4B and C). However, there was damage at the phloem tissue in chilled fruit, which resulted in the collapse and flattening of these areas (Fig. 4E, H, and N; Supplementary Fig. 1). This region was examined in detail. Unlike chilled fruit, neither TS nor IB was present in senesced fruit (Fig. 4A), and the tissues of the VB in non-chilled and senesced fruit were similarly healthy with no damage to the phloem in any of the sections examined (Fig. 4F and G, I and J, and L and M). There was a high co-occurrence of TS and phloem collapse, regardless of tissue location (Fig. 4A, H, K, and N). In most cases, the xylem elements and sclerenchyma fiber around the VB were unaffected by chilling. The specificity of phloem deterioration was clear: it occurred only in chilled fruit, neither in non-chilled nor in senesced tissue.

The early changes in phloem integrity that precede the appearance of TS in cold-stored TST were investigated. In this experiment, TST pineapple fruit kept at 10 °C for 21 days showed 10% TS, whereas the fruit stored for less than 21 days did not show TS (data not shown). Then, the degree of phloem damage during this chilled period was categorized as follows: (i) "None Detectable" (Fig. 5(1)A), (ii) "Mild," i.e., loss of phloem cell integrity but no collapse (Fig. 5(1)B–C), and (iii) "Severe," i.e., collapse of at least 50% of the phloem tissue (Fig. 5(1)D). The result showed that, at day 0 and in the senesced tissue (fruit kept at 25 °C for 14 days), only 25% of all VBs showed mild damage of the phloem (Fig. 5(2)). After 7 days at 10 °C, the percentage of VBs that showed mild damage increased from 25 to 90%, with the remainder starting to show severe damage. After 14 days storage, severe phloem damage increased from 10 to 40%, even though no TS were observed. After 21 days at 10 °C, TS were visible at the base of the fruit and, in this region, all VB showed phloem damage with more than 70% classified as severe. In addition, when the non-affected tissue adjacent to the TS region was observed, a similar proportion of phloem collapse was found (Fig. 5(2)). These results clearly suggest that phloem collapse occurs before TS, and that the phloem is the site of chilling injury.

3.2.2.2. Varietal differences in VB anatomical structure.
The anatomical structures of VBs from the four pineapple cultivars were compared. In IB-occurring cultivars, the only tissues studied were those presenting an incidence of PCI, i.e., TS. It was clear that, in TST, SV, and PTV, the phloem lost its integrity and buckled, particu-
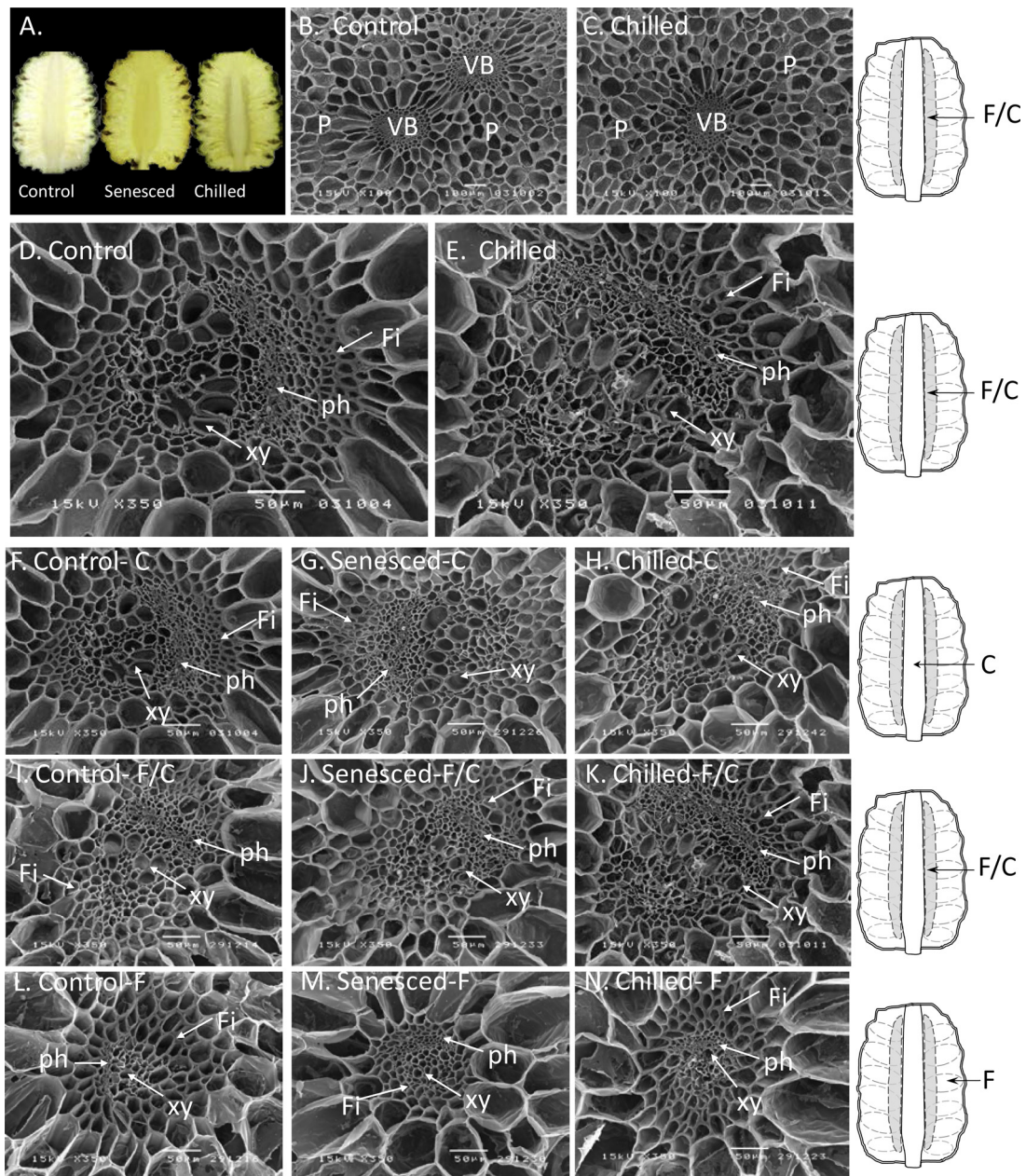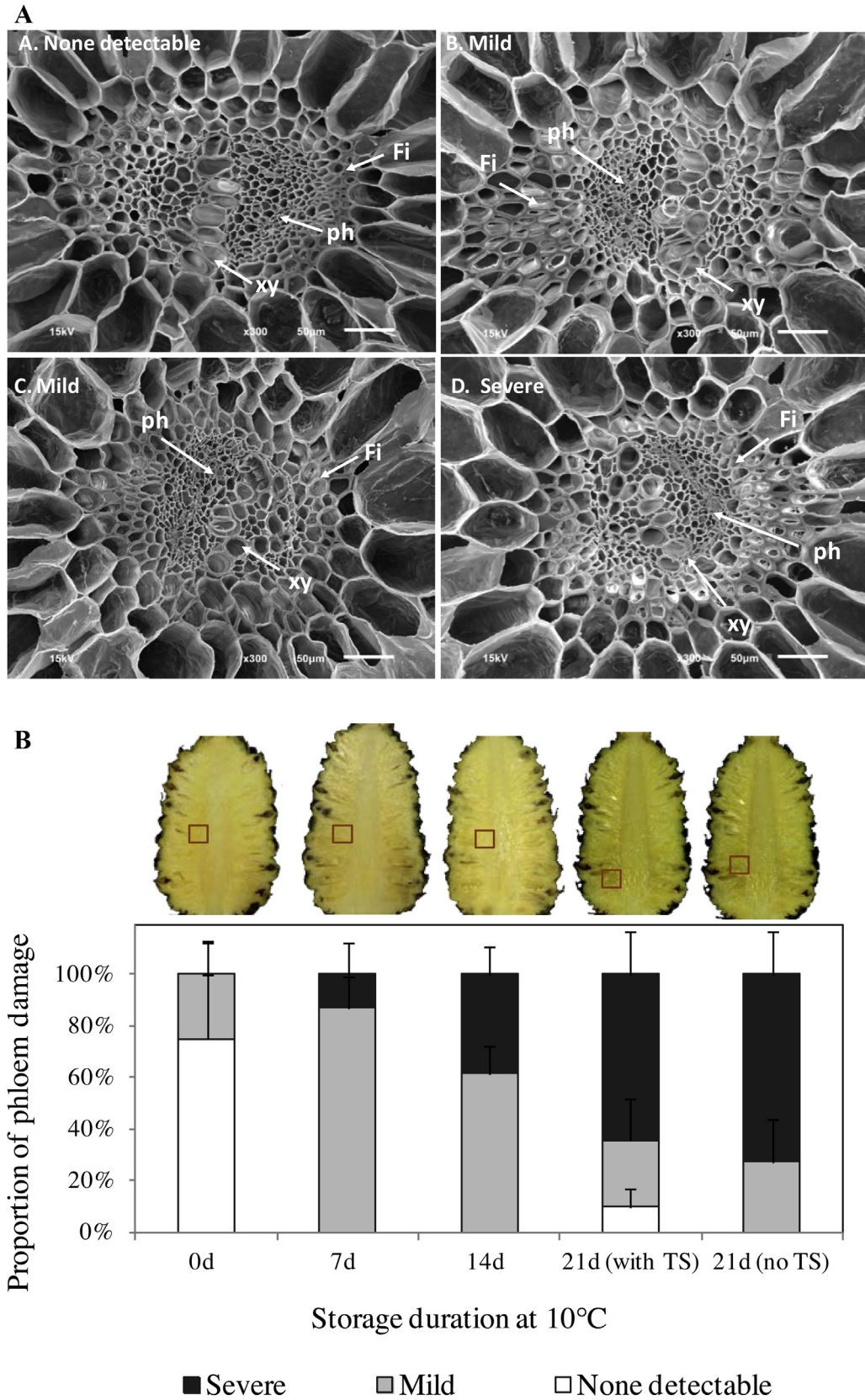
Fig. 4. Scanning electron micrographs showing the collapse of the phloem in the translucent tissue of TST pineapple. Samples of the non-chilled control, senesced, and chilled fruit were compared. (A) The tissue was examined at harvest (control), after storage at 25 °C for 14 days (senesced), and after storage at 10 °C for 14 days (chilled). Translucent symptoms (TS) were present in the chilled fruit but absent in control and senesced fruit. For the chilled fruit, only regions showing translucency were further examined. The VBs and adjacent non-VB parenchyma cells of control (B, D) and chilled fruit (C, E) were compared (D and E represent the higher magnification, i.e., 40× of B and C, respectively). The VBs at the C region of the control (F), senesced (G), and chilled fruit (H); the VBs at the F/C region of control (I), senesced (J), and chilled fruit (K); and the VBs at the F region of control (L), senesced (M), and chilled fruit (N) were compared. C = core region, F/C = flesh adjacent to core region, F = flesh region, P = non-VB parenchyma, Fi = sclerenchyma fiber, ph = phloem, xy = xylem. Bar scale = 100 μm (B and C), and = 50 μm (D–N).

larly in areas where TS was evident (Fig. 6A–C). The sclerenchyma fiber layer of the MD2 cultivar was thicker, and was almost twice as large as that in the other cultivars, resulting in VBs that were approximately two-fold larger in size in this cultivar (Fig. 6D; Supplementary Fig. 1E and F). Not only was the size different, but the arrangement was also distinct. In MD2, the sclerenchyma fiber formed a concentric ring completely surrounding the xylem and phloem. In other words, in MD2, there were multiple layers of the sclerenchyma cells on the lateral sides of VB. In contrast, in PTV, there were no more than two layers of the sclerenchyma cells on the lateral sides of VB. In TST, the sclerenchyma fibers did not completely surround the xylem ring, leaving the xylem elements close

to the non-VB parenchyma cells (Fig. 6; Supplementary Fig. 2). In severely chilled fruit as the browning symptoms developed, the 'torn-apart' area on the lateral sides of the VB at the phloem was always observed (Supplementary Fig. 1C).

3.2.2.3. Anatomical structure of VB of other species. For comparative purposes, we included SEM data from chilled mango, avocado, and banana in our study because changes in VBs due to PCI have been fairly established in these species (Murata 1969; Woolf 1997; Phakawatmongkol et al., 2004; USDA, 2004; Woolf et al., 2005). However, no report on changes specific to the phloem in relation to CI has been documented in any of theses studies. PCI symptoms

Fig. 5. (1) Scanning electron micrographs of TST pineapple tissues that differ in their severity of phloem damage. The degree of phloem damage at the F/C (the flesh adjacent to core) from fruit kept at 10 °C for 0, 7, 14, and 21 days was determined. Damage was categorized as (i) "None detectable" (A), (ii) "Mild," i.e., loss of phloem cell integrity but no collapse (B and C), and (iii) "Severe," i.e., collapse of at least 50% of the phloem tissue (D). Fi = sclerenchyma fiber, ph = phloem, xy = xylem. Bar scale = 50 μm. (2) The severity of phloem damage of TST pineapple fruit kept at 10 °C for 0, 7, 14, and 21 days. Damage was categorized as "None detectable," "Mild," and "Severe," respectively, (as described in Fig. 5(1)). The VB tissues used to determine the severity of damage were counted from 3 fruit. The TS were observed in fruit kept at 10 °C for 21 days but were not observed in fruit kept at 10 °C for 0, 7, and 14 days. Both TS tissue and the adjacent non-TS region from fruit kept at 10 °C for 21 days were examined for phloem damage.
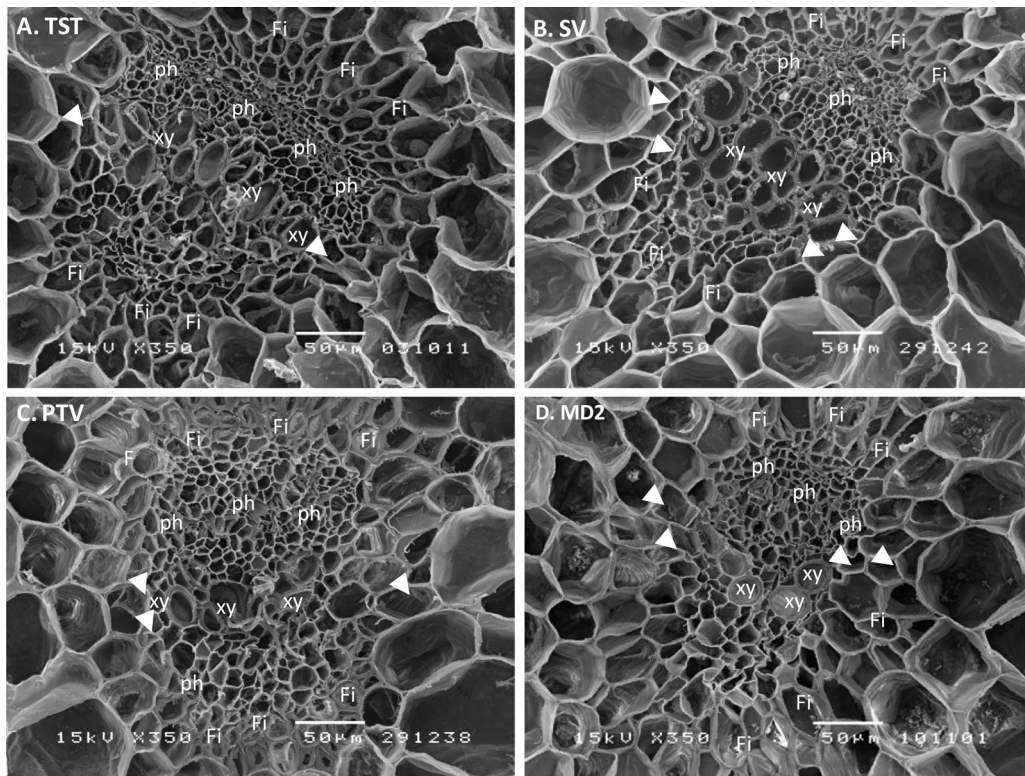
Fig. 6. Scanning electron micrographs of VBs from four pineapple cultivars that differ in their severity of internal browning symptoms (IB) caused by chilling injury. The TS tissue from the F/C region was examined in all IB-affected fruit. (A) The IB-susceptible cultivar TST fruit was examined after two weeks storage at 10 °C. A single layer of the sclerenchyma cell on the lateral sides of the VBs was observed (arrow head). (B and C) IB-moderately tolerant cultivars SV and PTV were examined after three weeks storage at 10 °C. There were double layers of the sclerenchyma cells on the lateral sides of VBs (arrow head). (D) The IB-resistant MD2 was observed after three weeks storage at 10 °C in the absence of translucency symptoms. There were multiple layers of the sclerenchyma cells on the lateral sides of VBs (arrow head), resulting in two-fold larger compared to other cultivars (Supplementary Fig. 1E and F). Fi = sclerenchyma fiber, ph = phloem, x = xylem. Bar scale = 50 μm.

were manifested as vascular browning of the pulp in avocado, dark-brown streaks of sub-epidermal tissues of the peel in banana, and browning in the fibrous part of the flesh directly around the seed coat in mango fruit (Fig. 7C, F, and I, respectively). For SEM examination therefore, tissues where PCI occurred, i.e., showing IB, were sampled from the pulp, peel, and flesh adjacent to the seed coat of avocado, banana, and mango, respectively. All chilled tissues sampled showed a collapse of phloem tissues (Fig. 7).

4. Discussion

Identifying the site of IB occurrence due to low temperature storage might help to better understand the mechanism of PCI in pineapple fruit and other similarly affected species. The present study uniquely demonstrates that IB in pineapple occurred at the phloem and different IB-sensitive cultivars showed differences in VB structure and organization. Similar results were obtained when the entire experiment was repeated 2–3 times.

4.1. The phloem is the site of IB in pineapple fruit

The current results clearly showed that the VB tissue of pineapple fruit was the site of H_2O_2 accumulation. There were also higher levels of phenolic compounds and PPO activity specifically due to chilling stress (Fig. 2). In addition, phloem damage was found only in the chilling-stress-induced translucent tissue (Figs. 5–7). There may be three explanations as to why TS selectively occurs at the phloem and why phloem collapse leads to TS. The first involves phloem properties, the second, phloem ultrastructure, and the third, changes in cell wall degradation of phloem tissue.

4.1.1. Phloem properties

The cells in the phloem tissue may contain different cellular properties, e.g., membrane composition or a different antioxidant system, compared to the cells in other tissues. To our knowledge, the cellular properties of phloem tissue have not yet been studied. However, some data supporting this idea was found in *Paspalum dilatum* Poir. After 3 days at 10 °C, ultrastructural change of the chloroplasts in the phloem parenchyma preceded that of the chloroplasts in the lower mesophyll. This different rate of change was also associated with the severity of damage of the upper mesophyll (Taylor and Craig, 1971).

4.1.2. Phloem ultrastructure

The phloem tissue contains sieve elements, companion cells, parenchyma cells, and in some cases includes: fibers, sclereids, and laticifers (Taiz and Zeiger, 2002). Transportation through the sieve tube occurs by pores in the sieve plates or sieve areas between the sieve tube elements. These pores allow the plasma membrane of a sieve tube element to be continuous with that of its neighboring sieve tube element (Taiz and Zeiger, 2002). Chilling induces the loss of cell turgor, vacuolization of the cytoplasm, and swelling disintegration of cell organelles (Kratsch and Wise, 2000). This will lead to leakage of the substrate needed by PPO for the synthesis of the brown substances as proposed by Woolf et al. (2005) for avocado. These browning-related substances could be conducted throughout the fruit via the continuous sieve tube connection. The collapse of the phloem would lead to the exudates from the VBs filling up the intercellular air spaces of the surrounding tissue, resulting in translucent spots (Hassan et al., 2011), and eventually, browning, as proposed for other species (Murata, 1969; Woolf, 1997; Woolf et al., 2005). After 21 days at 10 °C, paradoxically, the non-TS and adjacent
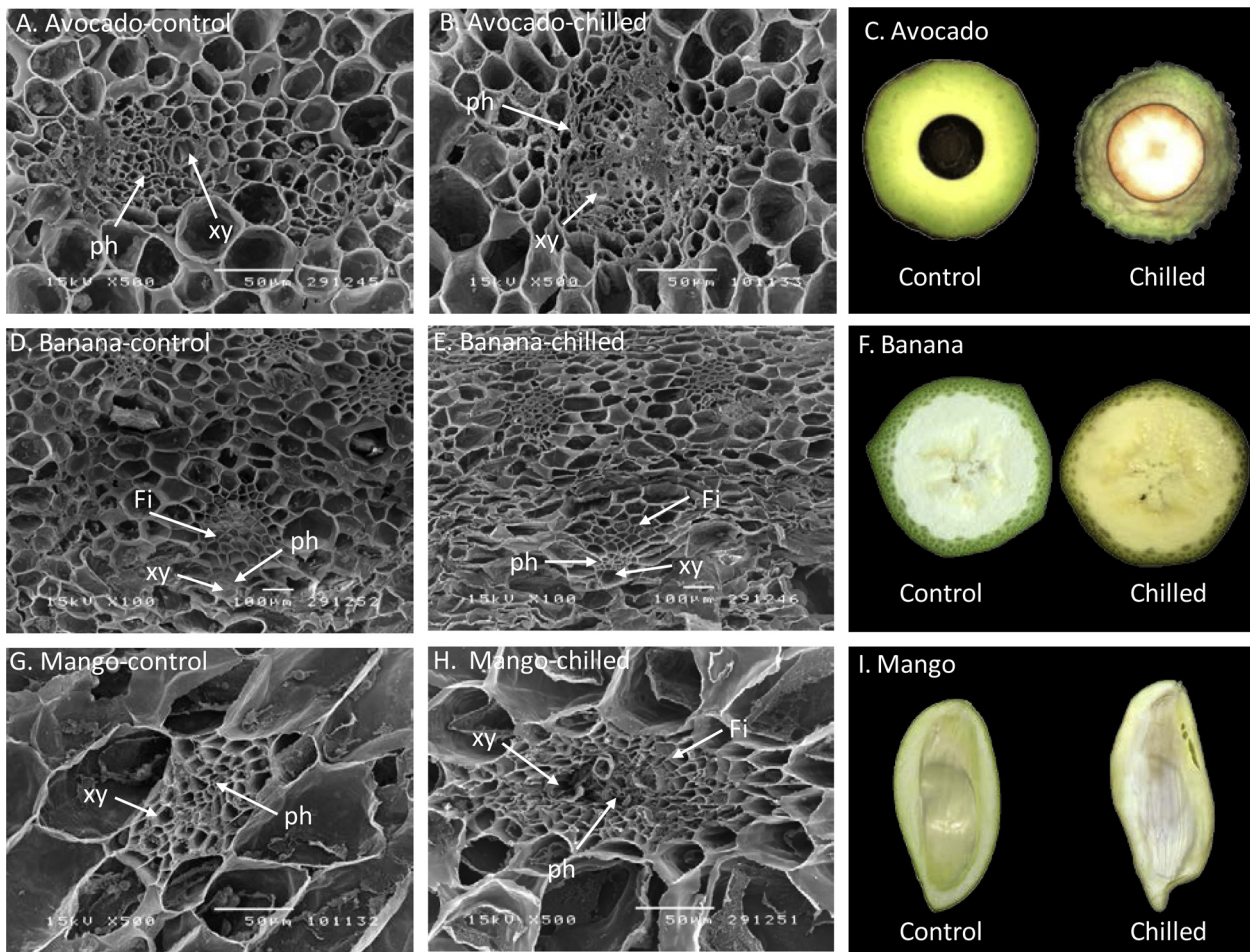
Fig. 7. Scanning electron micrographs of VBs and images of control and chilled avocado, banana, and mango fruit. Chilled fruits showed a dark brown color at the vascular bundles of the pulp (C; avocado), peel (F; banana), and flesh adjacent to the seed coat (I; mango). The phloem of chilled fruits (B, E, and H) showed evidence of collapse as compared with that of non-chilled fruit, which did not (A, D, and G). Fi = sclerenchyma fiber, ph = phloem, x = xylem. Bar scale = 50 μm (in A, B, G, H) and 100 μm (in D, E).

TS regions had a similar proportion of phloem collapse. This may be explained by variation in the number of layers of sclerenchyma cells at the lateral side of the VBs in these regions. Non-TS regions have more fiber layers, which effectively restrict phloem exudates to the surrounding parenchyma tissue, preventing the intercellular air spaces from filling with liquid. The opposite would be true for the TS-regions, which have fewer layers, allowing for a greater flow of exudates to the intercellular spaces and the rapid appearance of TS. Only after a longer period of chilling stress, would the protective function of the sclerenchyma fibers in the non-TS tissue become less effective, leading eventually to TS.

4.1.3. Phloem cell wall

Degradation of the cell wall might play a role in phloem collapse upon IB induction. The cell wall composition of the phloem differs from other tissues (Taiz and Zeiger, 2002). The walls are non-lignified and are weaker in structure compared to the tracheary elements of the xylem (Taiz and Zeiger, 2002). Each sieve tube element is interconnected with the metabolically active companion cells by plasmodesmata, which act as cytoplasmic bridges (Verma and Hong, 2001). In addition, around the plasmodesmata, there is a deposition of callose (β-1, 3-glucan). As maturation of sieve element progresses, the callose and the middle lamella cell wall material in this region are degraded to form a plate structure with widened pores (Truernit et al., 2008; Lichtenberger et al., 2014). In citrus, chilling causes pitting and browning of the flavedo and induces the activity of β-1, 3-glucanases (Sanchez-Ballesta et al.,

2006). The capability of this enzyme in degrading callose, which is deposited in the plasmodesmata in sieve element, might play a role in the phloem collapse upon chilling in pineapple.

Alternatively, it is possible that the non-VB parenchymal cells located next to the VB tissue might be the sites of IB initiation (Hu et al., 2012). However, the production of H_2O_2 and phenolic compounds, and higher PPO activity in the translucent tissue were detected specifically in the VB tissues, not in other living non-VB cells after chilling (Fig. 2). These data indicated that IB did not randomly occur in any living parenchyma cell but is specific to the phloem. Therefore, if the phloem is the site of IB initiation in PCI of pineapple fruit, then future studies should focus on how different traits of the phloem, e.g., membrane properties, antioxidant system, etc., vary among cultivars differing in IB response.

4.2. Why do IB symptoms occur at the flesh/core (F/C) boundary of pineapple fruit?

In all IB-occurring cultivars studied, the VBs found at the F/C boundary contained fewer sclerenchyma fiber cells than did the VBs found at the C region, except in the PTV cultivar (Fig. 3). The sclerenchyma fibers are composed of lignified cells, which offer protection to the VBs (Taiz and Zeiger, 2002). When these anatomical features of the pineapple are considered, the link between VB number and the appearance of IB at the F/C is clear. This occurrence may be explained by the morphological structure of the pineapple fruit. The fruit is a composite of multiple individual fruitlets, fused
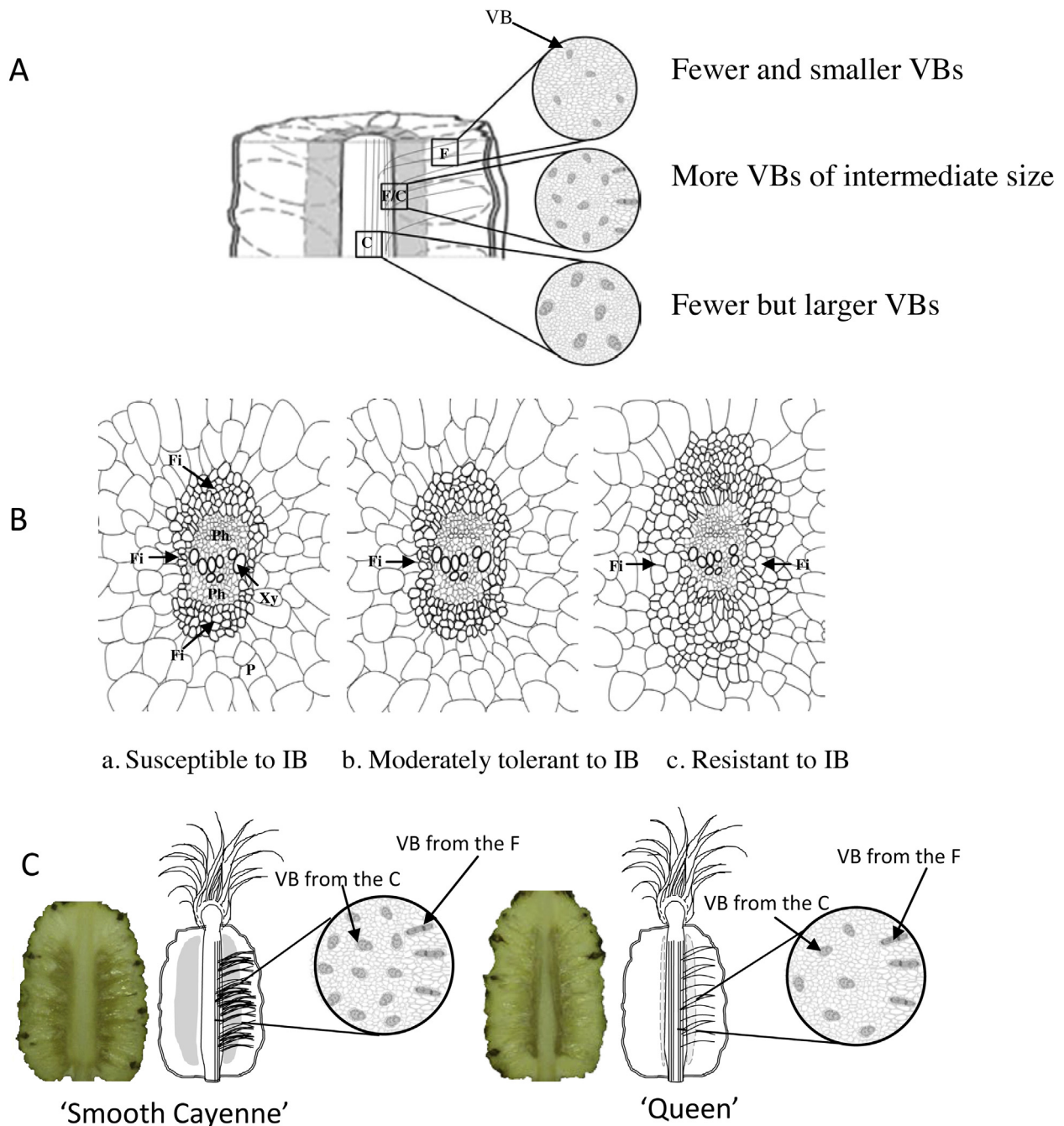
Fig. 8. A model to describe VB structure in relation to IB of pineapple fruit. (A) In this image, the VBs were vertically arranged at the core and horizontally at the F. The insert image shows VB size and number in the C, F/C, and F regions. At the F/C region of the pineapple fruit, a higher number of VBs was observed compared to the other regions. (B) The VBs of the F/C region reproduced from micrographs of: a. TST, b. PTV, and c. MD2 (Supplementary Fig. 2). The VBs in this region of the IB-resistant MD2 contained a greater proportion of sclerenchyma fiber, resulting in larger VBs. In MD2, there were multiple layers of the sclerenchyma cell on the lateral sides of the VBs. In contrast, PTV and SV had no more than two, and TST had only a single layer of the sclerenchyma cell on the lateral sides of the VBs. (C) In the 'Smooth Cayenne' group, i.e., PTV, IB spreads outwards from the periphery of the C towards the F so that symptoms are mostly seen in the F. In the 'Queen' group, i.e., TST and SV, IB increases inwards from the periphery of the C, towards the C center, so that symptoms are seen in both the C and F/C. The vertical and horizontal lines within the fruit represented the VB organizations. The density of VBs at the F/C of the 'Smooth Cayenne' group was higher than that of the 'Queen' group. C = core region, F/C = flesh adjacent to core region, F = flesh region, Fi = sclerenchyma fiber, ph = phloem, xy = xylem.

to each other and to the C. At maturity, the fruitlets become the edible fleshly part of the fruit composed predominantly of parenchyma cells and some VBs containing lignified cell walls (Supplementary Fig. 2). The C of the fruit consists mainly of VBs with connections to the stem. The C region constitutes a stele of VB, one connected to the other vertically. From the C region, the VBs are distributed to the F/C of the fruit (Okimoto, 1948). The VBs in the F/C region form the

denser network that grows both vertically and horizontally toward the F regions (Fig. 8A). These data suggested that the higher number of VBs at the F/C regions might explain why IB symptoms are easily detectable to the naked eye. However, as mentioned previously, the properties of phloem, e.g., their membrane properties and antioxidant system, at the F/C region might play a role in IB development.

4.3. The VB organization in IB-resistant MD2

Consistent with published data, the IB-resistant MD2 showed no IB and TS (IB score 0%) after being stored at 10 °C for three weeks (Table 1; Raimbault et al., 2011). It is possible to hypothesize that differences in vascular structure may play a role in the observed lack of IB in resistant cultivars. Among all four cultivars studied here, the number and size of the xylem elements and phloem per VB did not correlate with varietal IB resistance (Table 1; Fig. 3C–E). It is noteworthy, however, that the IB-resistant MD2 had fewer VBs but more sclerenchyma fiber cells surrounding the phloem and xylem, resulting in a larger size of VBs compared with other cultivars. In addition, the number and size of VBs and the number of sclerenchyma cells in all regions of the MD2 fruit were similar (Fig. 3A, B, and F). In MD2, there were the multiple layers of the sclerenchyma cells on the lateral sides of VB. In contrast, in PTV and SV, there were no more than two layers of the sclerenchyma cells on the lateral sides of VB, and only a single one in TST. These special anatomical features may serve a protective purpose, preventing separation of the VBs themselves and limiting further damage to the tissue from chilling injury (Fig. 8; Supplementary Fig. 2C and D). Interestingly, MD2 stored at 10 °C for six weeks exhibited chilling injury at the exocarp (data not shown). These data suggested that there are potentially different physiological and biochemical processes occurring in the fruit of the IB-resistant MD2 including: (a) different physiological and biochemical properties of phloem cell (as mentioned in Section 2), (b) fewer metabolically active phloem cells due to the lower number of VBs compared to the susceptible lines (Fig. 8B), and (c) very low levels of PPO activity or phenolics so that any browning compounds produced were not visibly detectable (Raimbault et al., 2011).

4.4. The difference in VB organization between the 'Smooth Cayenne' group (PTV) and the 'Queen' group (TST and SV)

Pineapple varieties have been grouped based on their clonal origin, and the progression of PCI has been reported to show differentiation that loosely correlates with these groupings (Nukulthornprakit and Siriphanich, 2005). Two of the cultivars used here, TST and SV, belong to the 'Queen' group, while PTV belongs to the 'Smooth Cayenne' group (Morton, 1987). Based on our observations and on other reports (Stewart et al., 2002), the earliest development of PCI symptoms in susceptible pineapple cultivars occurs around the F/C. The symptoms then spread inwards towards the C in the 'Queen' group (i.e., TST and SV cultivars) and outwards to the F region in the 'Smooth Cayenne' group (i.e., PTV cultivar) (Stewart et al., 2002). The number of VBs in the F and C regions of both pineapple groups was similar; however, the number of VBs at the F/C boundary of the PTV cultivars was higher than that of the 'Queen' group (Fig. 3A).

5. Conclusion

In summary, this study is the first to report that collapsed phloem tissue is the site of postharvest IB occurrence in pineapple fruit. The VB tissues had the highest activity of PPO and highest levels of H_2O_2 and phenolic compounds as detected by histological assays. A higher incidence of IB symptoms at the F/C in pineapple fruit may result from the relatively higher number of VBs and a reduction in the relative amount of the protective sclerenchyma fiber in the F/C region compared to other regions. A number of the VB at the F/C regions observed in pineapples classified as the 'Smooth Cayenne' type was higher than that in the more susceptible fruit of the 'Queen' group. The IB-resistant MD2 fruit had fewer numbers of VBs, but a higher number of sclerenchyma fiber layers, resulting in a larger VB size.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.07.011.

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