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Polyphenol Oxidase in Pawpaw (*Asimina triloba* [L.] Dunal) Fruit Pulp from Different Varieties

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Abstract

Pawpaw (*Asimina triloba* L. Dunal) is a tree fruit from the tropical Annonaceae family. Pawpaw currently has very limited commercial production because of its high perishability. Polyphenol oxidase (PPO) is responsible for enzymatic browning in pawpaw pulp. The objective of this research is to characterize PPO extracted from different pawpaw varieties. With respect to PPO activity, six of the varieties (Taytwo, Rebecca's Gold, NC-1, Overleese, Rappahannock, and Green River Belle) exhibited PPO activity that was statistically higher than Quaker's Delight and Lynn's Favorite. The other four varieties (SAA Zimmerman, Shenendoah, KSU-Atwood, IXL) exhibited PPO activity that was not significantly different from each other or Quaker's Delight and Lynn's Favorite, but were significantly lower than Taytwo, Rebecca's Gold, and NC-1. Kinetic parameters (Vmax, Km) and their ratio can be used to relate enzyme velocity with substrate affinity. Varieties that exhibited a high ratio, i.e. a very active enzyme due to a high Vmax and/or a low Km, are Rebecca's Gold, Taytwo, NC-1, and KSU-Atwood). The results presented indicate that certain varieties exhibit conditions that suggest PPO could have a lower inherent impact on tissue browning, especially Lynn's Favorite, Green River Belle, IXL, SAA Zimmerman, and Overleese. On the other hand, certain varieties (Taytwo, Rebecca's Gold, NC-1, and perhaps KSU Atwood) exhibit PPO activity, Vmax, and Km values that suggest inherently high PPO activity and thus increased potential for browning. Overall, understanding PPO activity may help to explain post-harvest discoloration of pawpaw pulp and aid the commercial selection of more shelf-stable varieties.

Keywords: pawpaw, polyphenol oxidase, enzymatic browning, Asimina

1. Introduction

Pawpaw (*Asimina triloba* L. Dunal) is a tree fruit from the tropical Annonaceae family. It is highly unusual in that it is one of only a handful of temperate species of the more than 130 tropical species in the Annonacea family. It is indigenous to the eastern United States. Pawpaw fruit flavor resembles a combination of banana and mango (R.G. Brannan, Salabak, & Holben, 2012) and has been shown to have a high polyphenolic content (Brannan, Peters, & Talcott, 2014; Harris & Brannan, 2009). However, pawpaw fruit pulp is susceptible to rapid post-harvest increases in soluble solids content, tissue browning, and fruit softening (McGrath & Karahadian, 1994). Research on the physiology and biochemistry of pawpaw fruit associated with ripening and postharvest storage is limited. Some post-harvest changes have been reported, such as the increases observed for respiration, ethylene generation (Archbold & Pomper, 2003), headspace volatiles, and soluble solids (McGrath & Karahadian, 1994). A change in the antioxidant profile of the fruit during ripening has also been reported (Harris & Brannan, 2009; Kobayashi, Wang, & Pomper, 2006, 2008).

Pawpaw currently has very limited commercial production because of its high perishability. Pawpaw is classified as a climacteric fruit, and it attains ethylene and respiratory climacteric peak within 3 days after harvest at ambient temperature (Archbold & Pomper, 2003). At the same time, the fruit softens rapidly such that within 5 days it becomes too soft for handling (Galli, Archbold, & Pomper, 2008). The shelf life of pawpaw fruit that is ripened on the tree has been reported to be from 2 to 3 days at room temperature (Layne, 1996) to 5 to 7 days at room temperature (Archbold, Koslanund, & Pomper, 2003) although this can be extended with refrigeration. Tissue softening is accompanied by tissue browning, which has been linked to the enzyme polyphenol oxidase (PPO), commonly associated with browning in fruits (Fang, Wang, Xiong, & Pomper, 2007).

PPO is responsible for enzymatic browning in fruits by catalyzing mono- and o-diphenol conversion to

o-quinones during ripening and postharvest handling, storage and processing. In Annonaceous fruits, PPO activity has been determined for atemoya (Chaves, Ferreira, Da Silva, & Neves, 2011), cherimoya (Prieto et al., 2007), soursop (Deoliviera, Guerra, Maciel, & Liveri, 1994; Falguera, et al., 2012), and sugar apple (Wu, 2000). Currently, there is a single published study on the activity of PPO in pawpaw (Fang et al., 2007). This work identifies two isoforms of pawpaw PPO (EC 1.10.31) of 28.2 and 38.3 kDa, with greatest activity at pH 6.5-7.0 and 5-20^{o}C. Substrates for PPO include many polyphenolic compounds that have been identified in pawpaw pulp (Brannan et al., 2014).

The activity of PPO can very among varieties. For example, different PPO isoforms have been reported in different papaya cultivars, with correspondingly different PPO activities (Shaw, Chao, & Chen, 1991). In the case of pawpaw, little is known about the differences between PPO activity and its susceptibility to discoloration among the more than 80 different varieties of pawpaw. Identification of PPO in pawpaw fruit pulp is important because the information will be valuable for the selection of varieties that are less susceptible to undesirable browning reactions. The objective of this research is to characterize PPO extracted from different pawpaw varieties.

2. Method

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Twelve commercial cultivars of pawpaw, hereafter referred to as varieties, were harvested from Fox Paw Ridge Farm, Cincinnati, Ohio. The whole pawpaw fruits were transported to the laboratory on ice and the descriptive characteristics (weight of each fruit, weight of the pulp from each fruit, color, % Brix, hardness, and pH) were measured as described below. Fruits from each variety were processed by hand to remove the skin and seeds and the pulp from each variety was pooled. Pulp from each variety was stored in polyethylene/nylon FoodSaver 27.94-cm bags (Jarden Corp., Rye, NY) with an oxygen transmission rate of 6.7 cc/m^2/24 h/23°C/0% RH and sealed immediately under vacuum until PPO analysis.

2.1 Descriptive Characteristics of the Pawpaw Fruit

After each whole fruit was weighed, a small piece of skin was removed to expose the pulp that was used to measure color, % Brix, hardness, and pH. A calibrated Konica-Minolta BC-10 was used to determine C.I.E. L*, a*, and b* values by placing the colorimeter in direct contact with exposed pulp. Juice (~1 ml) was extracted directly from the pulp and % Brix recorded directly using a refractometer. Penetrometry (hardness) was performed using a TA.XT2 Texture Analyzer (10-mm-diameter cylindrical probe, solid platform, crosshead speed 5 mm/s, depth 10 mm) and reported as kg of force. The pH of each fruit was recorded using a calibrated pH meter inserted directly into the pulp. After these measurements were taken, the weight of the pulp from each fruit was recorded and the fruit yield measured as the weight of pulp compared to the weight of the whole fruit including skins and seeds.

2.2 Polyphenol Oxidase (PPO) Measurement

Crude enzyme extracts from pawpaw were used to determine PPO activity based on the slope of the enzymatic assay standardized to protein content. The Michaelis-Menten constant [Km] and maximum velocity [Vmax] were determined. Specific conditions for crude enzyme extract, protein content, and the PPO assay are described in detail below.

2.2.1 Preparation of Crude Enzyme Extracts

Crude enzyme extracts were prepared in triplicate for each of the varieties according a published method specific to pawpaw (Fang et al., 2007). Pawpaw pulp was mixed with 0.2-M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.5) containing 5% (w/v) polyvinylpolypyrrolidone (PVPP), 2% (w/v) Amberlite XAD-4 and 2% (v/v) Triton X100 in a 2:3 (g/ml) ratio. After centrifugation at 18,000 x g for 20 min at 4^{o}C, the supernatant, which contained the crude PPO, was kept at 0^{o}C until protein and enzyme activity measurements were performed on the same day.

2.2.2 Lowry Protein Assay

Soluble protein in the supernatant was determined using the Lowry method. Crude extracts were mixed with Lowry Reagent (2% Na_2CO_3 in 0.1N NaOH, 1% $CuSO_4(5H_2O)$, and 2% $KNaC_4H_4O_6(4H_2O)$), then with 50% Folin & Ciocalteu's (FC) phenol reagent. After 30 min, absorbance at 750 nm was monitored. Protein was quantified based on a standard curve prepared from pure albumin.

2.2.3 Polyphenol Oxidase (PPO) Enzyme Assay

PPO activity is the increase in absorbance over time when catechol, a PPO substrate, is added to the crude extracts. Pawpaw crude enzyme extract and 2 M phosphate buffer at pH=6.5 were mixed for 5 minutes, after

which catechol solution (0.2%) was added. Absorbance at 420 nm was monitored every 10 seconds for 60 seconds. The slope of the increase in absorbance was calculated and PPO enzyme activity reported as ΔABS/min/g protein.

2.3.4 Determination of Kinetic Parameters

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined by varying the concentration (0.05 - 0.4%) of catechol in the assay procedure described above. A Lineweaver-Burke plot was drawn and the kinetic parameters (Vmax) and (Km) determined.

2.3.5 Experimental Manipulations or Interventions

Means were calculated for the descriptive characteristics of the pawpaw fruit. PASW (v. 18, Chicago, IL) was used to analyze PPO activity, hardness, and color (L*, a*, b*) using the general linear model procedure. The level of significance for all tests was set at 0.05. Means separations were achieved according to Duncan's multiple-range test.

3. Results and Discussion

The study was conducted in two parts. First, four known varieties of pawpaw, Green River Belle (GRB), Sue (SUE), Sunflower (SF), and Susquehanna™ (SQ) and wild fruit (WILD, i.e. not from a named variety) initially were screened for certain physical and chemical characteristics and PPO activity. Hereafter, WILD pawpaws will be discussed as a distinct variety. Then, PPO activity and kinetic parameters were determined on twelve pawpaw varieties: Green River Belle (GRB), IXL (IXL), KSU-Atwood™ (ATW), Lynn's Favorite (LF), NC-1 (NC1), Overleese (OL), Quakers Delight (QD), Rappahannock™ (RAP), Rebecca's Gold (RG), SAA Zimmerman (SAAZ), Shenandoah™ (SH), and Taytwo (T2).

3.1 Descriptive Characteristics of Five Varieties of Pawpaw Fruit

The number of individual fruits harvested from each variety ranged from 11 (SF) to 23 (SUE). The average whole fruit weight was highly variable, in the order of SQ (208 g) > GRB (187 g) > SF (152 g) > WILD (111 g) > SUE (106 g). In spite of this, there was no significant difference in pulp yield which ranged from 47-55% (Table 1).

Table 1. Pulp yield, C.I.E. color values (L*, a*, b*), hardness, pH, °B, and polyphenol oxidase (PPO) activity in four commercial cultivars and wild pawpaw pulp

Pawpaw Variety	Pulp Yield (%)	L*	a*	b*	Hardness	pH	°Brix	PPO Activity (ΔABS/min/g protein)
Green River Belle	49	50.3	-4.8	30.9 ^{a}	124 ^{b}	6.3	20 ^{b}	5.31 ± 0.68 ^{b}
Sue	47	52.6	-7.7	28.4 ^{a}	339 ^{a}	6.3	15 ^{c}	3.55 ± 1.09 ^{c}
Sunflower	48	64.8	-2.8	27.8 ^{a}	107 ^{b}	6.2	20 ^{b}	8.54 ± 0.72 ^{a}
Susquehanna™	55	52.9	-3.3	19.5 ^{b}	353 ^{a}	5.9	28 ^{a}	5.02 ± 1.90 ^{b}
Wild	50	56.1	-8.9	33.2 ^{a}	457 ^{a}	6.1	22 ^{b}	4.45 ± 0.65 ^{bc}

3.2 Color and Texture of Five Varieties of Pawpaw Fruit

The color of the pulp was quantified based on CIE chromaticity coordinates (L*, a*, b*), shown in Table 1. No differences were observed for lightness values (L*). Pulp from all varieties exhibited a negative a* values indicating that the pulp was "greenish" as opposed to "reddish," and no significant differences were exhibited. SQ exhibited a significantly lower b* value than the other four varieties, indicating that it was less yellow. Overall, the CIE chromaticity coordinates were in good agreement with previous research on pawpaw pulp (Brannan et al., 2014).

Pulp hardness values for pawpaws from the five varieties are reported in Table 1. There were significant differences among varieties on the order of WILD = SQ = SUE > GRB = SF in spite of the fact that each fruit was deemed ripe by applying slight pressure to the fruit while on the tree. Slight variations in fruit hardness at the same stage of ripening have been reported (McGrath & Karahadian, 1994) and described as being typical of differences in fruit development in fruits from trees.

3.3 PPO Activity of Five Varieties of Pawpaw Fruit

PPO activity of five varieties of pawpaw fruit are shown in Table 1. Significant PPO activity was observed in the order of SF > GRB > SQ ≥ WILD ≥ SUE. All varieties exhibited pulp pH in the range of 5.9-6.3 (Table 1). Previous research has shown that pawpaw pH exhibits maximum activity at pH 7.0, values in the pH 6-7 range exhibit high activity (Fang et al., 2007). In addition to having the lowest PPO activity, variety SUE had the lowest sugar content (15%) compared to the other varieties (Table 1). However, there was no clear trend with respect to sugar content and PPO activity in the other varieties.

3.4 PPO Activity of Twelve Varieties of Pawpaw Pulp

PPO activity among 12 varieties is shown in Table 2. Of the varieties studied in each of the two phases of this study, GRB was the only variety in common with both. The pH values of the varieties, also reported in Table 2, ranged from 6.1 to 6.8, which is in the high range of pawpaw PPO activity, as described in section 3.3. The PPO activity of GRB was in good agreement between the two phases of the current research, exhibiting PPO activity (ΔABS/min/g protein) of 5.31 (Table 1) and 5.36 (Table 2). PPO activity among the 12 variations showed that six of the varieties (T2, RG, NC1, OL, RAP, and GRB) exhibited PPO activity that was statistically higher than QD and LF. The other four varieties (SAAZ, SHEN, ATW, IXL) exhibited PPO activity that was not significantly different from each other or QD and LF, but were significantly lower than T2, RG, and NCl.

Table 2. Polyphenol oxidase activity (PPO), kinetic parameters (Km and Vmax), and pH of twelve commercial culitvars of pawpaw pulp

Variety	Activity (ΔABS/min/g protein)	Vmax (M/s)	Km (M)	Ratio V_{max}/K_m	pH
Taytwo	6.99 ± 0.32 [a]	0.020	2.27×10^{-2}	98	6.5
Rebecca's Gold	6.75 ± 1.96 [a]	0.021	2.71×10^{-2}	126	6.8
NC-1	6.30 ± 0.95 [a]	0.018	1.77×10^{-2}	73	6.1
Overleese	5.80 ± 1.80 [ab]	0.012	2.94×10^{-2}	15	6.5
Rappahannock™	5.71 ± 2.78 [ab]	0.014	5.15×10^{-2}	12	6.2
Green River Belle	5.36 ± 0.70 [abc]	0.017	4.63×10^{-2}	29	6.6
SAA Zimmerman	4.42 ± 0.81 [bcd]	0.011	1.77×10^{-2}	20	6.5
Shenandoah™	4.36 ± 0.47 [bcd]	0.015	2.82×10^{-2}	31	6.7
KSU-Atwood™	3.97 ± 0.47 [cd]	0.015	1.13×10^{-2}	61	6.5
IXL	3.69 ± 1.24 [cd]	0.016	4.17×10^{-2}	23	6.8
Quakers Delight	3.42 ± 1.30 [d]	0.014	1.81×10^{-2}	38	6.5
Lynn's Favorite	2.84 ± 0.57 [d]	0.010	5.29×10^{-2}	8	6.2

3.5 Kinetic Parameters of PPO from Twelve Varieties of Pawpaw Pulp

Shown in Table 2 are the kinetic parameters Vmax and Km for each of the twelve varieties sampled. Vmax, the maximum enzyme velocity, can be used to compare how fast PPO catalyzes its reaction in each of the varieties. The varieties that exhibit the highest PPO activity, T2, RG, and NC1, also exhibit the highest Vmax values of 0.20, 0.21, and 0.18 (M/s), respectively. LF is one of the varieties with the lowest PPO activity and exhibited the lowest Vmax of the varieties (0.10 M/s). Aside from these four varieties, there appear to be no discernible trends among the other eight, as some have exhibit higher PPO activity and lower Vmax (e.g. OL) and others with lower PPO activity and higher Vmax (e.g. IXL).

Km, the Michaelis constant, describes the substrate concentration (M) at which the enzyme exhibits half of its maximal velocity (Vmax). These values are reported in Table 2. Lower Km values indicate a high affinity for the substrate. LF, which exhibits the lowest PPO activity and Vmax, also exhibits the lowest Km. Aside from this observation, no other trends with respect to the kinetic parameters are evident. Lineweaver-Burk plots, which display the kinetic parameters for each variety, are shown in Figure 1.

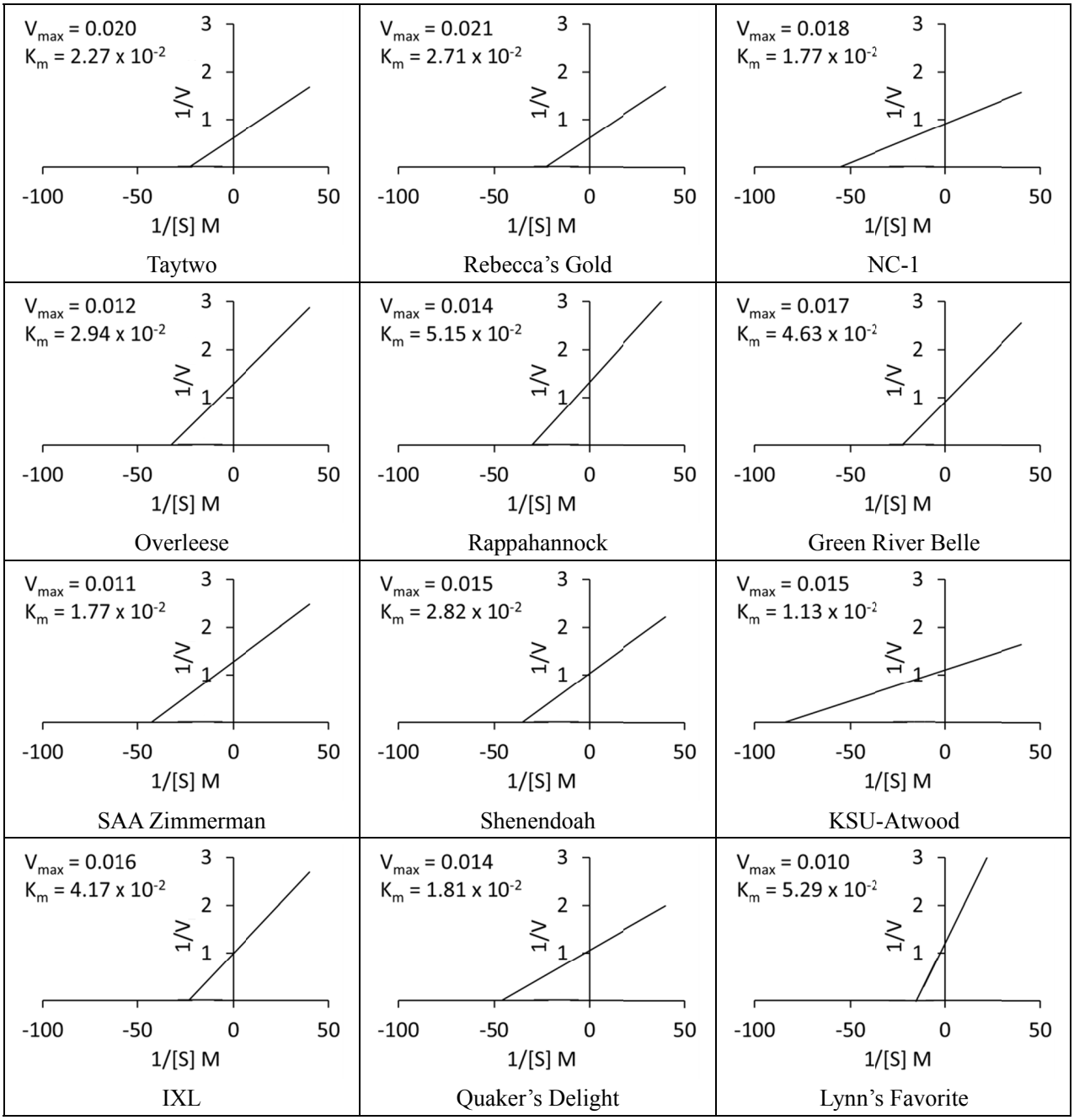

Figure 1. Lineweaver-Burk plots of PPO extracted from pulp of twelve pawpaw varieties

The ratio of Vmax to Km can be used to relate enzyme velocity with substrate affinity. A high ratio indicates a very active enzyme due to a high Vmax and/or a low Km. As shown in Table 2, varieties can be classified into two groups, with a group of varieties with ratios above 60 (RG, T2, NC1, ATW) and the remaining varieties with ratios below 30. Of the four varieties with values above 60, three (RG, T2, NC1) appear to be driven by high Vmax values and intermediate Km values, whereas the high ratio for ATW appears to be driven by its low Km value, the lowest of any variety.

Taken together, these results indicate that certain varieties exhibit conditions that suggest PPO could have a lower inherent impact on tissue browning. Chief among these varieties is LF which exhibited low activity, low Vmax, and high Km. Other varieties that should be considered are GRB, IXL, SAAZ, and OL. On the other hand, certain varieties, RG, T2, NC1, and perhaps ATW, exhibit PPO activity, Vmax, and Km values that suggest inherently high PPO activity and thus increased potential for browning.

4. Summary and Conclusion

PPO activity in this study was derived using catechol (IUPAC name: benzene-1,2-diol) as a substrate because the initial characterization of pawpaw PPO used this molecule (Fang et al., 2007). Catechol is a small diphenol that is found in small concentrations in many fruits so it is a potentially relevant substrate in fruits such as pawpaw. However in a separate study, our group has found that larger molecules such as epicatechin-based procyanidins predominate in pawpaw pulp (Brannan et al., 2014). Therefore, differences in PPO activity and its kinetic parameters may represent differences due to the affinity of PPO to catechol rather than between varieties of pawpaw PPO in its native conditions. Characterization of PPO activity with substrates more likely to be found in higher concentrations in the fruit may yield different information.

In order to increase its potential for commercialization, the pawpaw exhibits challenging post harvest properties. Its climacteric peak that occurs within 3 d after harvest coincides with major deteriorative changes in the fruit, chief among them the enzymatic discoloration attributed to PPO. Factors that can affect PPO by inhibiting or retarding its activity should be explored, potentially in fruit that exhibit low PPO potential as described above (LF, GRB, IXL, SAAZ, OL). Some of these factors include novel processing techniques that could inhibit or retard PPO activity, such as high pressure processing, and the addition of chemical inhibitors.

Overall, understanding PPO activity may help to explain post-harvest discoloration of pawpaw pulp and aid the commercial selection of more shelf-stable varieties.

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