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Comparison of phenylpropanoid metabolism of three physiological disorders in apple and pear

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Abstract: Apples and pears are the main temperate fruit tree species, and metabolic disorders may occur during their development and post-harvest storage periods, leading to physiological diseases. In this study, we detected the phenylalanine ammonia-lyase (PAL) enzyme and related metabolites involved in phenylpropanoid metabolism in the pulp of pear with hard-end or cork spot and in the pulp of apple with bitter pit. These the three physiological disorders differed in phenylpropanoid metabolism and had similar PAL activity, but their polyphenol, flavonoid, and lignin contents changes were completely. For fruits with the three types of physiological metabolic disorders, the auxin content in the tissues with metabolic disorder symptoms were higher than that in the healthy tissues. In summary, the three physiological disorders showed the similar changes in the activities of key enzymes (i.e., PAL) involved in phenylpropane metabolism, but their metabolites significantly differed.

Keywords: auxin; hard-end; cork spot; bitter pit; flavonoid; lignin

Fruit growth, ripening, and senescence are complex processes controlled by multiple developmental and environmental signals (Dar et al. 2018). Metabolic changes occurring in ripe or senescent fruits during post-harvest storage lead to a general deterioration in quality attributes, namely decreased flavor and 'off-aroma' compound generation (Pott et al. 2020). Various fruits can experience metabolic disorders during development and after harvesting, leading to physiological disorders and significant losses to the fruit industry (Thompson 2010).

Apples and pears are the two most important fruits grown in temperate regions (Retamales 2011), and China ranks first in their production and acreage. Notably, both fruits are prone to physiological disorders, such as bitter pit in apples and cork spot in pears, during ripening and storage. These two physiological disorders are commonly observed in the apple and pear industries worldwide (Yahia et al. 2019; Qiu et al. 2020). The symptoms of apple bitter pit are small dark depressions near the calyx end of the fruit caused by the col-

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lapse of flesh cells just below the peel (Jemrić et al. 2016; Qiu et al. 2020). The symptoms of pear cork spot are observed several weeks before harvest: affected fruits develop sunken areas darker green than unaffected parts of the fruit (Bevacqua 1992). Cork spot symptoms are similar to those of apple bitter pit; thus, another name for this disorder is pear bitter pit (Faust, Shear 1969).

Another physiological disorder is hard-end disorder, observed in pears in many pear-growing regions worldwide. The first symptom of hard-ended fruit is abnormal green or yellow coloration in the fruit. This symptom is observed when the fruit has developed to one-third or half of its size or during the end of the flowering period. Owing to the slow development of the surrounding tissues, obvious calyx protrusions tend to form (Faust, Shear 1969).

The mechanism underlying these three physiological disorders is unknown (Thompson 2010). However, research has suggested that all three symptoms of physiological imbalance are due to the lack of Ca^{2+} in fruits or metabolic disorders of fruit cells caused by lower Ca/K, Ca/K + Mg, and Ca/N concentration ratios than that in healthy fruits (Wang et al. 2018; Qiu et al. 2020). Notably, Ca^{2+} supplementation during agricultural production has not effectively reduced the incidence of these disorders (Raese 1989; Torres et al. 2017). Thus, the formation mechanisms of these three disorders are complex (Dar et al. 2018), and studying fruit Ca^{2+} alone will not explain their formation mechanisms (Wang et al. 2021).

Plant metabolism plays a crucial roles in fruit quality, yield, and disease resistance (Beauvoit et al. 2018), and different metabolites have different biological functions (Dixon et al. 2006). Metabolites reflect physiological changes (Tang, Wang 2006). In a targeted metabolomic analysis of apples, the contents of 15 phenolic compounds in flesh with bitter pit were higher than those in healthy flesh (Zupan et al. 2013). In our wide target metabolomics analysis on apples with bitter pit, the phenylpropanoid synthesis pathway was the most significant metabolic process, and the flavonoid content in the flesh of bitter pit fruit was significantly higher than that in healthy flesh (Huang et al. 2023).

Phenylpropane metabolism is a critical pathway in plant secondary metabolism (Li et al. 2021). Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropane metabolism pathway, and the lignin produced in this pathway is a cell wall component that affects plant growth, development, and stress resistance (Liu et al. 2018). Flavonoids regulate fruit color, eliminate reactive oxygen species in cells, and improve fruit quality (Farooq et al. 2020). PAL activity is positively correlated with the cellular content of lignin, phenolic compounds, and flavonoids in cells (Medda et al. 2020). Lignin has been extensively studied in research on the hard-end and cork spot of pears (Wang et al. 2018; Liu et al. 2023), but the content of polyphenols and flavonoids has not been reported. Therefore, in this study, we conducted biochemical analyses of PAL activity and lignin and flavonoid substances in the three aforementioned physiological disorders hard-end, cork spot and bitter pit to study their similarities and differences and to provide technical support for further research on their formation mechanisms and prevention.

MATERIAL AND METHODS

Plant material. This study used 'Cuiyu' and 'Qiuyue' pears, grown in Jining, Shandong Province, and 'Fuji' apples, grown in Yantai, Shandong Province, China. The 'Fuji' apple is the main variety of apple worldwide, and in China, it accounts for 60% of the total apple cultivation area, with an area of 125.3×10^4 hm^2. 'Qiuyue' pear is one of the main varieties in China and Japan, with a cultivation area in China of 2.7×10^4 hm^2. 'Cuiyu' pear is one of the earliest maturing pear variety in China, with a cultivation area of 1.3×10^4 hm^2.

The 'Cuiyu' pears were ready for harvesting on July 10, and from those fruits, those hard-end were selected. The 'Qiuyue' pears were ripe and harvested on September 10, and from those fruits, those with cork spot were selected. The 'Fuji' apples were harvested on October 20, and from those fruits, those with bitter pit were selected.

Extraction and Assays of PAL. PAL extraction was performed as previously described (Ballester et al. 2006). PAL was then inactivated by adding a 6 mol/L hydrochloric acid solution to the reaction mixture. PAL activities were determined as previously described (Babaoğlu Aydaş et al. 2013).

Lignin, phenolic, flavonoid, and auxin contents. Take 2 g of each flesh, grind it under liquid nitrogen, put it into 80% alcohol solution, centrifuge (12 000 rpm) for 10 minutes and take the supernatant for the analysis of lignin and flavonoids. Lignin content was analyzed using a previously described meth-

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od (Nayak et al. 2020). The total flavonoid content of the pulp was measured using aluminum chloride ($AlCl_3$) according to the spectrophotometric method, using quercetin as a standard (Win et al. 2019). Take 2 g of each flesh, grind it under liquid nitrogen, put it into 80% methanol solution, centrifuge (8 000 rpm) for 10 min and take the supernatant for the analysis of the phenolic content. The phenolic content was determined using the Folin-Ciocalteu method (Ainsworth, Gillespie 2007). Auxin was commissioned for testing by a commercial service provider (Standard Testing Group Co., Ltd. Qingdao, China).

Statistical analysis and graphics software. Data were analyzed to calculate the mean and standard deviation by using GraphPad Prizm 7.0 (GraphPad Software, Boston, MA, USA), based on Tukey's test, a multiple comparison method suitable for treatment groups with equal sample sizes. The advantages of the Tukey test for this study are that it is easy to perform, requires relatively small samples for the experiment, and the confidence level of its test results can reach 95% (Sawilowsky 2014). We randomly selected 15 healthy fruits and 15 fruits with physiological disorders. Each of the five fruits were used as one replicate for a total of three replicates. Data are expressed as means ± SD (n = 3). Software (GraphPad Prizm 7.0) was used for statistical mapping. The results of the data analysis are shown as scatter plots with bars (Figures 2– 6).

RESULTS

PAL activity detection for the three physiological disorders. The symptoms of three types of physiological disorders were examined. The flesh colour of the pear cork spot and apple bitter pit was darkened and brownish compared with that of the healthy flesh (Figure 1A and B), and flesh colour of the pear hard-end was the same as that of did the healthy flesh (Figure 1C). PAL activity in the cork spot of pear was 10.26 times that of healthy fruits. PAL activity in the bitter pit of apple was 3.64 times that in healthy fruit. The difference in hard-end was minimal for example, 1.83 times in PAL activity between the hard-end of pears and that of healthy fruits (Figure 2). This result suggests that the formation of pear cork spot, hard-end, and apple bitter pit is directly related to PAL activity and that the occurrence of these three physiological disorders would lead to an increase in PAL activity.

Comparison of lignin content. Lignin synthesis is derived from phenylalanine in phenylpropanoid metabolic pathways (Vanholme et al. 2019). The lignin content in pear cork spot and hard-end was significantly higher than that in healthy fruits, and the lignin content in apple bitter bit was significantly lower than that in healthy fruits (Figure 3). The content of lignin in the affected tissues pear hard-end was consistent with that in pear tissues affected with cork spot; that is, the lignin content in the metabolically disordered part increased significantly. This result indicates a direct correlation between the formation of lignin and pear hard-end and cork spot.

Comparison of polyphenol content. Plant polyphenols are a class of plant components that contain multiple hydroxyphenol groups and are widely present in plants (Cheynier et al. 2015). The polyphenol content in the pear hard-end site was lower than that in the healthy site, but the difference was not significant, and the polyphenol content in the pear cork spot and apple bitter pit sites was significantly higher

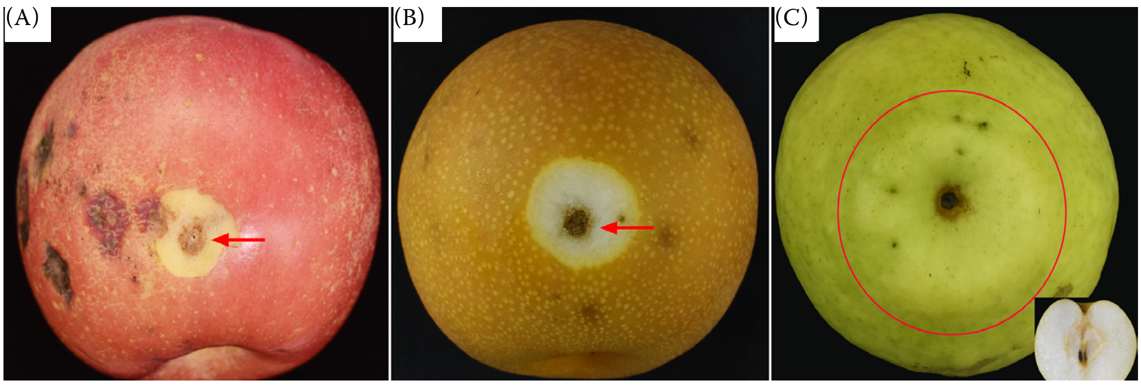

Figure 1. Symptoms of three physical disorders: (A) apple bitter pit, red arrow points to the site of bitter pit; (B) pear cork spot, red arrow points to the site of cork spot; (C) pear hard-end, thee site of hard-end is in the red circle. The inner picture is longitudinal section anatomy of pear hard-end. The scar bar is 4 cm

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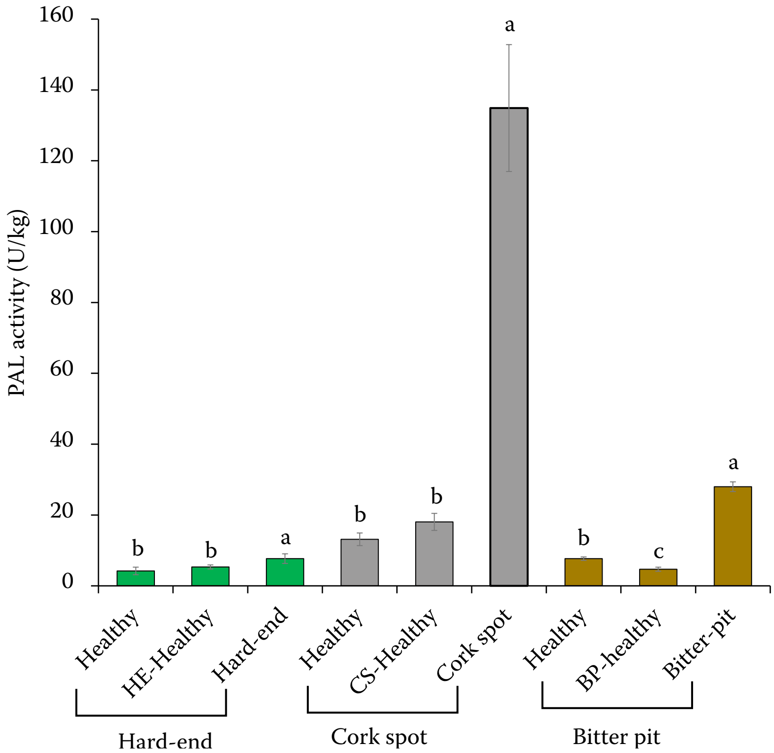

Figure 2. A comparison of PAL activity in healthy fruit flesh and fruit flesh of fruit with metabolic disorders

Healthy – the flesh of healthy fruit; HE-Healthy – the healthy flesh of fruits with hard-end; Hard-end – hard-end-affected tissues; CS-Healthy – the healthy flesh of fruits with cork spot; Cork spot – the flesh of cork spot; BP-Healthy – the healthy flesh of fruits with bitter pit; Bitter-pit – bitter-pit-affected flesh; letters above boxes indicate significant differences between fruit pulp types at the $P < 0.05$ level

than that in the healthy fruit (Figure 4). Flavonoids are polyphenolic compounds found in plants. The flavonoid content in the pear hard-end site was significantly lower than that in the healthy site; however, the flavonoid content in the pear cork spot and apple bitter pit sites was significantly higher than that in the healthy fruit (Figure 5). This result suggests the formation of pear cork spot and apple bitter pit is due to high flavonoid content.

Comparison of auxin content. Liquid chromatography analysis demonstrated that the auxin content in the metabolic disorders symptoms (pear hard-end and apple bitter pit) was higher than that in healthy fruit, and the auxin content

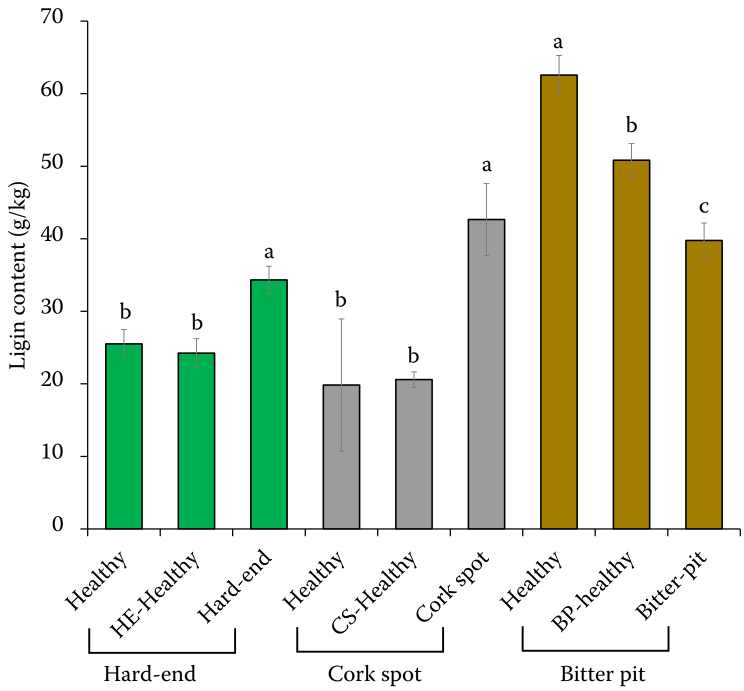

Figure 3. A comparison of lignin content in healthy fruit flesh and fruit flesh with metabolic disorder

Healthy - the flesh of healthy fruit; Hard-end – hard-end-affected tissues; CS-Healthy – the healthy flesh of fruits with cork spot; Cork spot – the flesh of cork spot; BP-Healthy – the healthy flesh of fruits with bitter pit; Bitter-pit – bitter-pit-affected flesh; letters above boxes indicate significant differences between fruit pulp types at the $P < 0.05$ level

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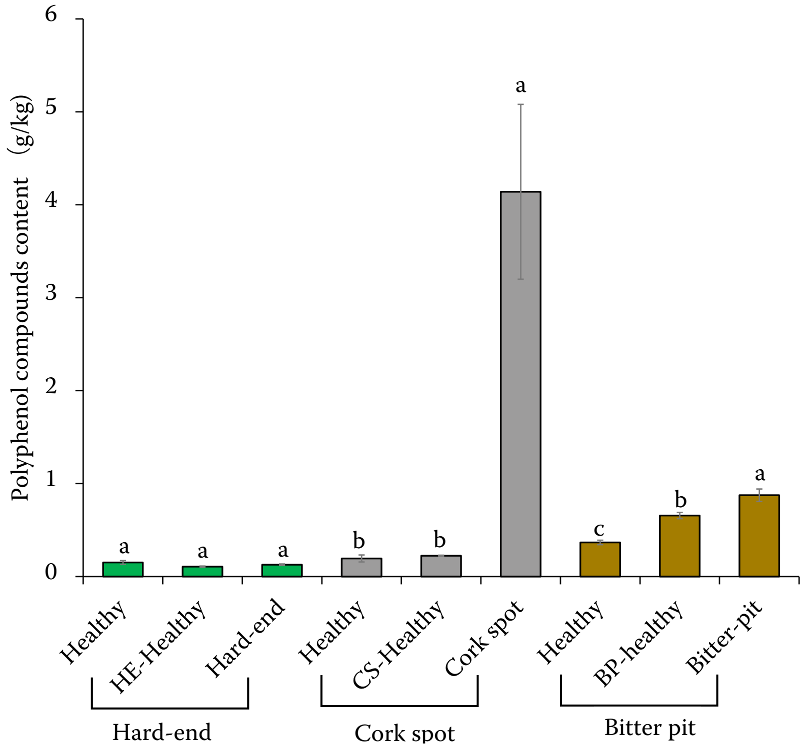

Figure 4. A comparison of polyphenol content in healthy fruit flesh and fruit flesh with metabolic disorder

Healthy – the flesh of healthy fruit; Hard-end – hard-end-affected tissues; CS-Healthy – the healthy flesh of fruits with cork spot; Cork spot – the flesh of cork spot; BP-Healthy – the healthy flesh of fruits with bitter pit; Bitter-pit – bitter-pit-affected flesh; letters above boxes indicate significant differences between fruit pulp types at the $P < 0.05$ level

in symptomatic tissu s of pear affected with cork spot was lower than that in healthy fruit. These three physiological metabolic disorders have a feature in common: the auxin content in the healthy flesh of fruits with metabolic disorders is lower than that in the metabolic disorders part (Figure 6).

DISCUSSION

Secondary metabolism is a quantitative study of metabolites in life systems that can reflect dynamic changes in endogenous (physiological and developmental) and exogenous (environmental) factors (Dix-

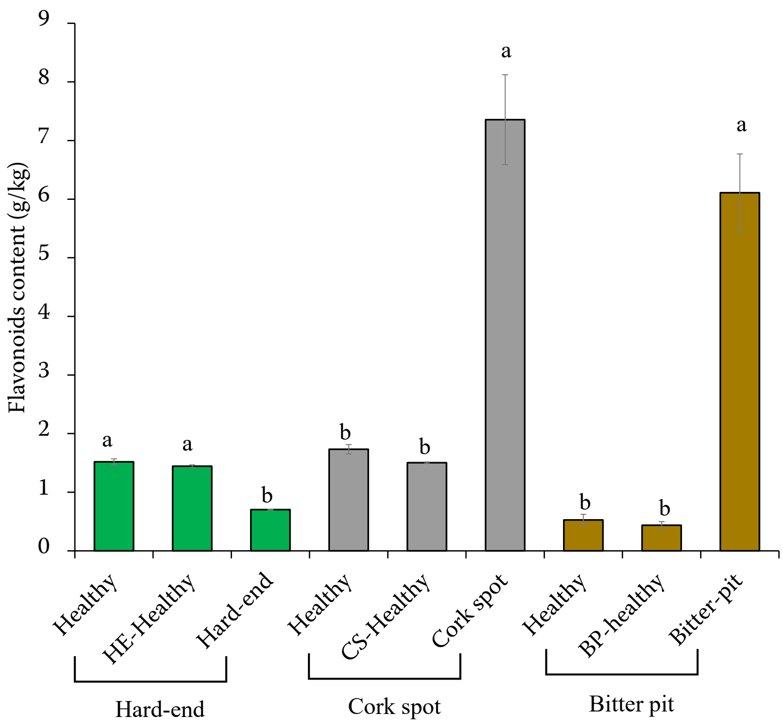

Figure 5. A comparison of flavonoid content in healthy fruit flesh and fruit flesh with metabolic disorder

Healthy – the flesh of healthy fruit; Hard-end – hard-end-affected tissues; CS-Healthy – the healthy flesh of fruits with cork spot; Cork spot – the flesh of cork spot; BP-Healthy – the healthy flesh of fruits with bitter pit; Bitter-pit – bitter-pit-affected flesh; letters above boxes indicate significant differences between fruit pulp types at the $P < 0.05$ level

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on et al. 2006; Tang, Wang 2006). Phenylpropanoid metabolism is an important secondary metabolic process in plants (Ge et al. 2023). PAL is the link between primary and secondary metabolism in plants, and its activity is regulated by biotic and abiotic factors (Barros et al. 2016; Isah 2019). The three physiological disorders examined in this study exhibited different phenylpropane metabolites but the same changes in PAL activity. Therefore, our hypothesis was that pear cork spots, hard ends, and apple bitter pits are related to phenylpropane metabolism. Salicylic acid (SA) reduced PAL activity and reduced the chilling injury of pomegranates (Sayyari et al. 2011) and tomato (Aghdam et al. 2014). Heat treatment reduced PAL activity in citrus, reducing CI (Sanchez-Ballesta et al. 2000). Ascorbate acid (ASA) treatment of loquat fruit reduced PAL activity, decreased lignin content, increased fruit juice content, and improved fruit quality (Cai et al. 2011).

In this study, the PAL activity in the flesh of pears with hard-end or cork spot and the flesh of apples with bitter pit was significantly increased. The lignin content in the flesh of pears with hard-end or cork spot significantly increased, consistent with results in the literature (Wang et al. 2018; Liu et al. 2023). Therefore, the incidence of these three physiological disorders could be reduced and the fruit quality could be improved by decreasing PAL activity during production. However, in this study, the lignin content of apple bitter pit was lower than that of the healthy pulp, which may be because the formation mechanism of apple bitter pit differs from that of the other two physiological disorders. Polyphenols and flavonoids have a deep colour (Dias et al. 2021; Mutha et al. 2021); thus, the flesh at bitter pit and cork spot sites is brown. The polyphenol content in the hard-end flesh was the same as that of the healthy flesh, and the flavonoid content was lower than that of the healthy flesh; thus, the flesh color of the hard-end fruit did not change. In summary, the formation mechanisms of these three physiological disorders differ.

Auxin and phenylpropane metabolism have a certain relationship (Peer, Murphy 2007). For example, Russet spotting (RS) is one of the most important post-harvest physiological disorders in iceberg lettuce (Ke, Saltveit 1988), and the application of exogenous auxin or 2, 4-D suppressed PAL activity and reduced RS scores (Ke, Saltveit 1986; Ritenour et al. 1996). However, in this study, auxin content and PAL activity in the physiologically disordered flesh were higher than those in healthy flesh, especially in hard-end and bitter pit fruits, and the auxin content and PAL activity in physiologically disordered flesh were higher than those in healthy fruits. We used transmission electron microscopy and observed that the Ca^{2+} concentration in bitter pit flesh was higher than that in

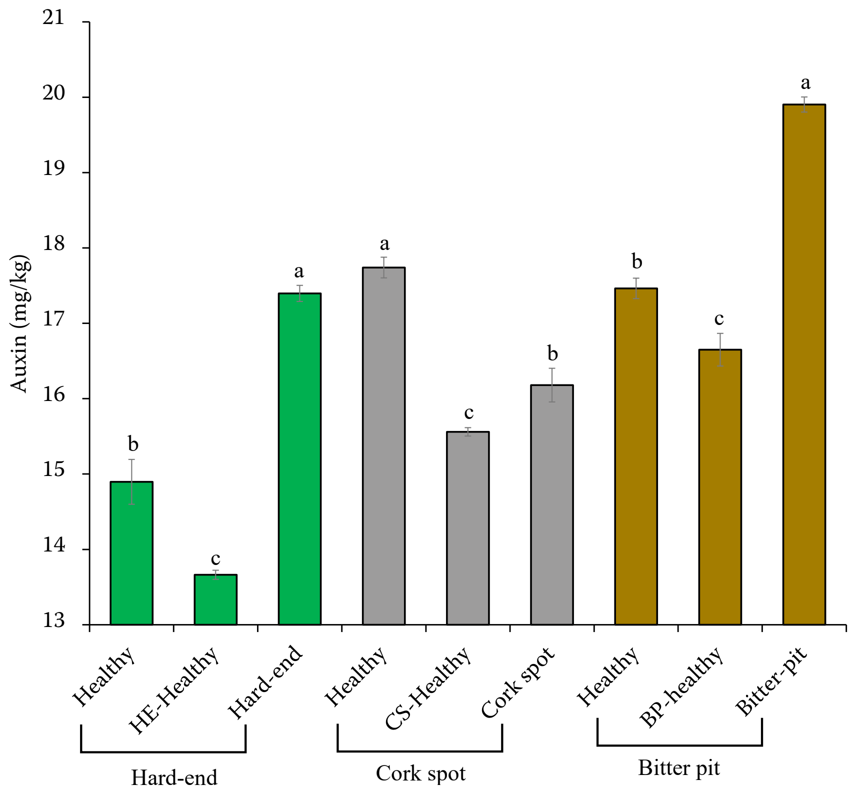

Figure 6. A comparison of auxin content in healthy fruit flesh and fruit flesh with metabolic disorder

Healthy – the flesh of healthy fruit; Hard-end – hard-end-affected tissues; CS-Healthy – the healthy flesh of fruits with cork spot; Cork spot – the flesh of cork spot; BP-Healthy – the healthy flesh of fruits with bitter pit; Bitter-pit – bitter-pit-affected flesh; letters above boxes indicate significant differences between fruit pulp types at the $P < 0.05$ level

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healthy flesh (Qiu et al. 2021). Notably, auxin inhibited Ca^{2+} efflux from apple cells (Huang et al. 2020) and increased cellular Ca^{2+} concentrations (Shishova, Lindberg 2004; De Vriese et al. 2019), which can increase PAL activity (Guo et al. 2015). This results may be a phenomenon that the metabolism of these three physiological disorders has in common.

CONCLUSION

The mechanism of formation of these three physiological disorders is not consistent based on the products of phenylpropane metabolism. The development and ripening of fruits are regulated by auxins, and fruits with these three physiological disorders show relatively consistent changes in auxin content and PAL activity. Therefore, further research should aim to determine the mechanism responsible for the increase in auxin in the physiological disorders of fruits, as well as the correlation between auxin and phenylpropane metabolism, to reduce the incidence of physiological disorders by regulating auxin.

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