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采后菠萝果实黑心病发病过程中乙醇代谢的变化

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摘 要:**【目的】**探究采后'巴厘'菠萝果实黑心病发生过程中乙醇代谢途径的变化。**【方法】**对六成熟(6 M)和八成熟(8 M)果实进行黑心病发病情况观察,并检测常温贮藏时,黑心病发病过程中果实乙醇代谢相关产物、代谢关键酶活性以及基因表达变化。**【结果】**随着贮藏时间的延长,菠萝黑心病病情指数逐渐提高,其中6 M果的病情指数显著高于8 M果($P<0.05$)。在贮藏前4 d,6 M果丙酮酸含量显著高于8 M果($P<0.05$)并呈下降趋势,而8 M果呈上升趋势;6 M果的乙醛含量为先上升后下降,第6天出现峰值,而8 M果为逐渐下降趋势。6 M果和8 M果乙醇含量变化趋势一致。丙酮酸脱羧化酶(pyruvate decarboxylase,PDC)和乙醇脱氢酶(alcohol dehydrogenase,ADH)活性变化呈先升后降趋势,且8 M果ADH活性显著高于6 M果,PDC和ADH基因表达与酶活性变化趋势一致。**【结论】**菠萝的采收成熟度与黑心病的发病率密切相关,成熟度高的8 M果贮藏过程中菠萝黑心病的发生率明显低于6 M果;伴随黑心病的发生,6 M果和8 M果的乙醇代谢相关产物、代谢关键酶活性存在差异,乙醇代谢可能与黑心病发生密切相关。

关键词:菠萝;黑心病;乙醇代谢

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The changes of ethanol metabolism in the pulp of pineapples during the postharvest incidence of blackheart disease

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Abstract:【Objective】Fermentation metabolism exists constantly in the pulp of pineapples, and the contents of metabolites (pyruvic acid, acetaldehyde and ethonol) and enzyme activities including pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are important indexes for evaluating the level of fermentation metabolism. Blackheart disease in pineapples [*Ananas comosus* (L.) Merr. 'Comte de Paris'] is a physiological disorder that may be induced by exposure to low temperature, either in the field or in postharvest storage and results in severe internal browning of pineapple fruit, However, the biochemical pathway of blackheart disease has not been clearly documented. In order to study the variation of fermentation metabolism in the process of pineapple blackheart disease, the pulp of pineapples from 6 mature (6 M) and 8 mature (8 M) fruits were investigated during storage at room temperature (25 ℃), respectively. 【Methods】The severity of pineapple blackheart disease was evaluated by assessing the ratio of the brown area in longitudinal transaction fruits. The content of pyruvic acid and the enzyme activities of PDC and ADH were determined using conventional physical and chemical analysis methods. The content of acetaldehyde and ethanol were measured by using a gas chromatograph, and the changes of gene expression of PDC and ADH were detected by real-time fluorescent quantitative RT-PCR. 【Results】The blackheart index increased gradually in the 6 M fruit and in the 8 M fruit during storage at 25 ℃, and the blackheart index in the 6 M fruit was significantly higher in the 8 M at 4 d of storage. The pyruvic acid content de-

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creased continuously in the 6 M fruit, while it increased to 16.68 μg·g^{-1} and then decreased gradually in the 8 M fruit when stored at 25 ℃. During 0 to 4 d of storage, the pyruvic acid content in the 6 M fruit was significantly higher in the 8 M fruit. Contrasting to the falling of acetaldehyde content in the 8 M fruit during whole storage at 25 ℃, the variation trend of acetaldehyde content in the 6 M fruit grew steadily from 0 to 6 d of storage and then declined rapidly, with the peak value reaching up to 23.31 μL·g^{-1}. And the acetaldehyde content in the 8 M fruit was notably lower than that in the 6 M fruit during the whole storage period at 25 ℃ except for 0 day. After increasing smoothly both in the 6 M and 8 M fruit from 0 to 4 d of storage, the ethanol content was sharply raised in the 8 M and slowly in the 6 M at the later stages, respectively. The ethanol content in the 8 M fruit grew to 124.35 μL·g^{-1} on the 10th day, which was significantly higher than that in the 6 M. The changes of PDC activities in the 6 M fruit and 8 M fruit stored at 25 ℃ in a similar manner, reached a plateau and then decreased, and grew slightly at the later stage of storage. The largest enzyme activities of PDC were 372.31 U·g^{-1} in the 6 M fruit on the 4th d and 385.46 U·g^{-1} in the 8 M fruit on the 6th day, respectively. The ADH activity in the 6 M fruit storage at 25 ℃ increased from 364.68 U·g^{-1} to 3 618.51 U·g^{-1} during the period from 0 to 6 d of storage, and then decreased to 270.82 U·g^{-1} protein on the 10th day. Likewise, the change of ADH activity in the 8 M fruit storage at 25 ℃ increased from 912.76 U·g^{-1} to 5 713.30 U·g^{-1} from 0 to 6 d of storage, and then decreased to 1 878.50 U·g^{-1} after 8 d of storage. The ADH activity in the 8 M fruit was higher than that in the 6 M fruit during the whole storage period. The relative gene expression of PDC in the 6 M fruit and in the 8 M fruit had an identical growth mode that increased during the earlier storage but decreased at the later storage, which were both consistent with the change of ADH activity. And they reached a peak of 3.19 on the 4th day and 8.90 on the 6th day, respectively. The relative gene expression of PDC in the 8 M fruit was significantly higher than in the 6 M fruit during the period from 6 to 10 d. As for the change of the expression level of ADH, it had a similarly changing pattern with the change of the PDC relative gene expression corresponding in the 6 M fruit and 8 M fruit, which grew sharply in the 6 M during 0 to 4 d and declined from 4 to 10 d of storage, and grew in the 8 M fruit during 0 to 6 d and declined later. ADH relative gene expression in the 8 M fruit is significantly higher than that in the 6 M fruit at the stage during 6 to 10 d of storage, as well as the maximum gene expression of ADH. As a result, the fermentation metabolism in pineapple fruit of the 8 M was more active than in that of 6 M.【Conclusion】The harvest maturity of pineapple pulp has a close correlation to blackheart in pineapple. And postharvest pineapple with a higher maturity has a more active fermentation metabolism but a lower blackheart incidence when stored at 25 ℃. The relationship between the ethanol metabolism and the incidence of blackheart disease remains to be further explored and researched.

Key words: Pineapple; Blackheart disease; Fermentation metabolism

菠萝黑心病是发生在菠萝果实中的一种生理病害[1]。在很多菠萝产区均有发生，包括中国[2]、斯里兰卡[3-4]、澳大利亚[5]、泰国[6]、巴西[7]、南非[8]等。黑心病通常发生在果实贮藏过程中，最初在果实上部和下部接近果心的小果基部出现半透明水渍状或浅褐色斑点，褐斑数量逐渐增多且向外扩散，最后形成大范围黑褐色组织，果实外部无明显症状，仍保持有正常成熟果特征，从外观很难判断果实是否发生黑心病[9-11]。据本实验室长期调查统计，采后‘巴厘’菠萝果实黑心病发病率超过90%。

目前，普遍认为黑心病，即果实内部褐变是由于多酚氧化酶将酚类物质氧化为醌类物质，进而累积形成的[12-13]。薛鑫等[14]认为当植物受到逆境胁迫（如低温）时，会在细胞中积累大量的活性氧，而果实代谢过程中产生的活性氧能促进酶促褐变[15]。Luengwilai等[16]认为在菠萝黑心病始发区域（接近果核的果肉区域）丰富的维管束组织及较少的厚壁纤维是发生黑心病的关键。Liu等[17]认为菠萝果实顶芽产生的内源ABA与菠萝黑心病发病率有关，并且内源ABA能有效降低黑心病发病率。至今为止，对于菠萝黑

心病的发病机制仍没有明确解释。本实验室已对菠萝黑心病发病前后相关基因表达谱进行了分析，发现乙醇代谢相关基因的表达与菠萝果实黑心病发生之间存在一定联系。因此，笔者利用生理生化和分子生物学技术，以‘巴厘’[*Ananas comosus*(L.)Merr. ‘Comte de Paris’]菠萝果实为试材，研究常温(25 ℃)贮藏时，六成熟(6 M)和八成熟(8 M)菠萝果实黑心病发生过程中乙醇代谢相关产物丙酮酸、乙醛、乙醇含量的变化及乙醇代谢关键酶丙酮酸脱羧酶(pyruvate decarboxylase，PDC)、乙醇脱氢酶(ethanol dehydrogenase，ADH)活性和相关基因表达的变化，探讨菠萝贮藏过程中，伴随黑心病的发生，乙醇代谢的变化。

1 材料和方法

1.1 材料与处理

供试菠萝于2015年10月采自广东省湛江市徐闻县菠萝试验基地。试验果采收后3 h送至海南省热带园艺产品采后生理与保鲜重点实验室(广东湛江)。挑选大小、质量(单果质量1 kg±50 g)相近，果表颜色均匀、无病虫害和机械损伤的果实，菠萝果肉糖酸比为(25±2)定义为六成熟(6 M)果，糖酸比为(30±2)定义为八成熟(8 M)果。试验用果平放在套有PE袋的镂空塑料篮里，恒定温度(25±0.5)℃，相对湿度85%~95%，分别于0、2、4、6、8、10 d切开菠萝观察黑心病发病情况，且每次取5个菠萝果实果轴中线木质部外围2~5 cm处果肉，混合，3次重复，立即用液氮冷冻并使用粉碎机打成粉末状，用50 mL保存管放置于-81 ℃冰箱中待用。

1.2 菠萝果实黑心病发生级数观察

根据张鲁斌等[18]的方法，将菠萝果实沿果轴中线切开观察，按黑心病病斑面积占剖面面积比例分为0~5等级。0级，无黑心、无变色；1级，出现黑心症状，黑心面积低于5%；2级，黑心面积占5%~10%；3级，黑心面积占10.01%~20%；4级，黑心面积占20.01%~50%；5级，黑心面积占50%以上。病情指数(disease index，DI)的计算公式如下：$DI=[\Sigma(Nx\cdot X)\cdot 100]/(5\Sigma Nx)$。式中X为发病级数，Nx为相应级别病果个数。

1.3 采后果实生理指标测定

1.3.1 丙酮酸含量测定 按照李红卫[19]的方法测定果肉中丙酮酸含量。结果以$\mu g\cdot g^{-1}$(以鲜质量计)表示，3次重复。

1.3.2 乙醛与乙醇含量测定 参照Zhang等[20]的方法并适当改动。取1 g样品加入3 mL蒸馏水静置5 min，于4 ℃ 10 000 $r\cdot min^{-1}$离心10 min，取上清液，再加入3 mL冰水，涡旋振荡沉淀，再次离心取上清液，合并2次上清液。将合并的上清液取5 mL注入20 mL顶空瓶中，加入2 g NaCl后密封顶空瓶，使用气相色谱仪测定。气相色谱条件：气相色谱仪安捷伦7890A(HP5柱子30 m×0.25 mm×0.25 μm)；进样口温度110 ℃，分流进样(分流比30∶1)；柱温50 ℃ 0.5 min，30 ℃·min^{-1}升到110 ℃，柱流量1 $mL\cdot min^{-1}$；检测器FID：温度250 ℃，N_2尾吹25 $mL\cdot min^{-1}$，H_2 30 $mL\cdot min^{-1}$，空气300 $mL\cdot min^{-1}$；CTC80进样器：烤箱105 ℃，5 000 $r\cdot min^{-1}$ 2 min，进样针83 ℃。3次重复，根据标准曲线计算乙醛和乙醇浓度，结果用$\mu L\cdot g^{-1}$(以鲜质量计)表示。

1.3.3 PDC和ADH活性测定 参照Ke等[21]的方法略作修改，测定PDC和ADH活性。取2 g样品，加10 mL 100 $mmol\cdot L^{-1}$ MES(pH=6.5)[其中含2 $mmol\cdot L^{-1}$二硫苏糖醇和1%(ρ)PVP]，于4 ℃ 14 000 $r\cdot min^{-1}$离心10 min，上清液分别用于PDC和ADH活性测定。PDC活性测定的酶反应体系包括3.2 mL 100 $mmol\cdot L^{-1}$ MES(pH=6.5)，0.5 mL 5 $mmol\cdot L^{-1}$硫胺素，0.5 mL 50 $mmol\cdot L^{-1}$ $MgCl_2$，0.5 mL酶提取液，0.1 mL乙醇脱氢酶(0.56酶活性单位)，0.2 mL 1.6 $mmol\cdot L^{-1}$ NADH，0.3 mL 50 $mmol\cdot L^{-1}$丙酮酸钠。加入丙酮酸钠后立即记时，用紫外分光光度计于340 nm处测定OD值变化。ADH活性测定的酶反应体系包括0.88 mL MES(pH=6.5)，0.06 mL 1.6 $mmol\cdot L^{-1}$ NADH，0.01 mL提取液，0.05 mL 80 $mmol\cdot L^{-1}$乙醛，加入乙醛后立即记时。根据Bradford[22]法，使用南京建成生物工程研究所提供的总蛋白定量测试盒测定样品总蛋白质含量，以牛血清蛋白作标准曲线。定义每min OD值变化0.001所需酶量为1个酶活力单位(U)，结果以$U\cdot g^{-1}$表示其相对酶活性变化。

1.4 PDC与ADH相对表达量测定

使用华越洋RNA试剂盒提取液氮粉样品总mRNA。基于笔者实验室前期对黑心病菠萝转录组和基因芯片分析，筛选出PDC与ADH差异表达基因，获得其碱基序列并设计引物，内参基因为菠萝管家基因γ-actin(HQ148720)，引物序列见表1，由上海生工生物技术有限公司合成。使用TOYOBO高

表1　引物序列

Table 1 Sequences of primers

基因名称 Gene name	引物 Prime sequences	产物长度 Products/bp
PDC	F:GTCTGTGTATGTATGCACTTCA	22
	R:CCAGAATATTAGGTATACTGTG	22
ADH	F:TTGGTGTCACCGACTTCGTGAA	22
	R:CAGCCATCATGAACACACTCAA	22
γ-actin	F:CTGGCCTACGTGGCACTTGACTT	23
	R:CACTTCTGGGCAGCGGAACCTTT	23

效率逆转录试剂盒在TaKaRa Standard PCR仪上对总mRNA进行逆转录获取cDNA。按照Thunderbird SYBR qPCR Mix（TOYOBO公司）试剂盒推荐体系，使用Light Cycler 480 Ⅱ型（Roche，France）荧光定量PCR仪对合成的cDNA进行定量分析。每个样品检测做3管平行试验，记录每个样品目的基因和γ-actin扩增的Ct值，根据各样品的Ct值，采用相对定量方法分析目的基因ΔCt值（ΔCt=目的基因Ct-内参基因Ct），根据ΔΔCt=试验组ΔCt-对照组ΔCt，计算$2^{-\Delta\Delta Ct}$。

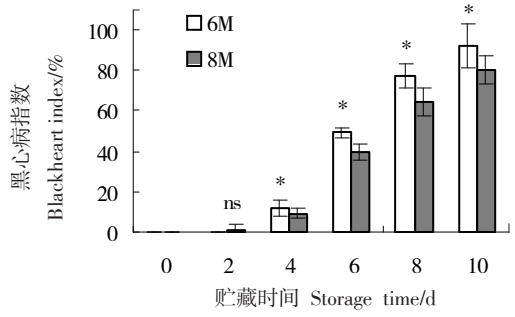
1.5　数据处理

试验数据采用Excel（2010）和OringinPro 8.5进行处理和作图，在SPSS v19上使用Duncan's新复极差法进行显著性分析。

2　结果与分析

2.1　常温贮藏过程中菠萝黑心病病情指数变化

如图1所示，常温贮藏过程中，6 M果在4 d时开始出现黑心病，而8 M果在2 d时出现黑心病，并且2者随时间变化，黑心病病情指数均逐渐升高。从4 d开始，6 M果黑心病病情指数始终显著高于8 M果（$P<0.05$）。



*表示在 $P<0.05$ 水平差异显著，ns 表示无明显差异。

* represents significant difference at $P<0.05$, ns represents no significant difference.

图1　常温贮藏过程中果实黑心病指数

Fig. 1 Blackheart index in fruit during storage at 25 ℃

2.2　菠萝黑心病发生过程中丙酮酸、乙醇和乙醛含量变化

由图2-A可知，在常温贮藏过程中，6 M菠萝果实丙酮酸含量呈下降趋势，而8 M果为先上升后下降，在第6天出现峰值，为16.68 μg·g^{-1}。在贮藏0~4 d时，6 M果的丙酮酸含量显著高于8 M果含量。

由图2-B可知，在常温贮藏过程中，6 M果乙醛含量呈先上升后下降的趋势，在第6天出现峰值，为23.31 μL·g^{-1}。而8 M果在贮藏过程中，乙醛含量逐渐降低，且含量均低于6 M果，并且在2~10 d时，乙醛含量显著低于6 M果。

从图2-C中可知，在常温贮藏过程中，6 M果和8 M果乙醇含量在0~2 d没有明显变化，从第4天开

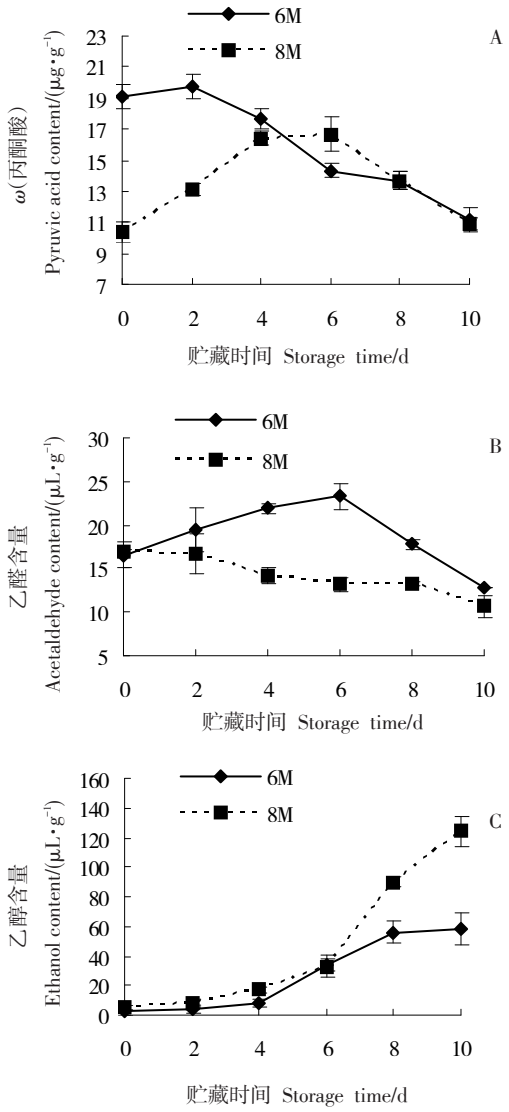


图2　果实在常温贮藏过程中丙酮酸、乙醛和乙醇含量变化

Fig. 2 The changes of pyruvic acid, acetaldehyde and ethanol contents in pulp during storage at 25 ℃

始呈上升趋势，且8 M果上升速度较快，在贮藏第10天时，乙醇含量达到124.35 μL·g^{-1}，显著高于6 M果的乙醇含量。

2.3 菠萝黑心病发生过程中PDC和ADH酶活性变化

如图3-A所示，在常温贮藏期间，在2种成熟度的菠萝果实中，PDC活性的变化趋势均为先上升后下降，其中6 M果在4 d时PDC活性达到峰值，为372.31 U·g^{-1}。8 M果在6 d时达到峰值，为385.46 U·g^{-1}。

如图3-B所示，在常温贮藏期间，2种成熟度菠萝的ADH活性均在0~6 d上升，在6 d时2者均达到峰值，6 M果ADH活性为3 618.51 U·g^{-1}，8 M果为5 713.10 U·g^{-1}。6 M果在6 d之后均开始下降，而8 M果在第8天之后出现下降。在整个贮藏期间8 M果的ADH活性均大于6 M果。

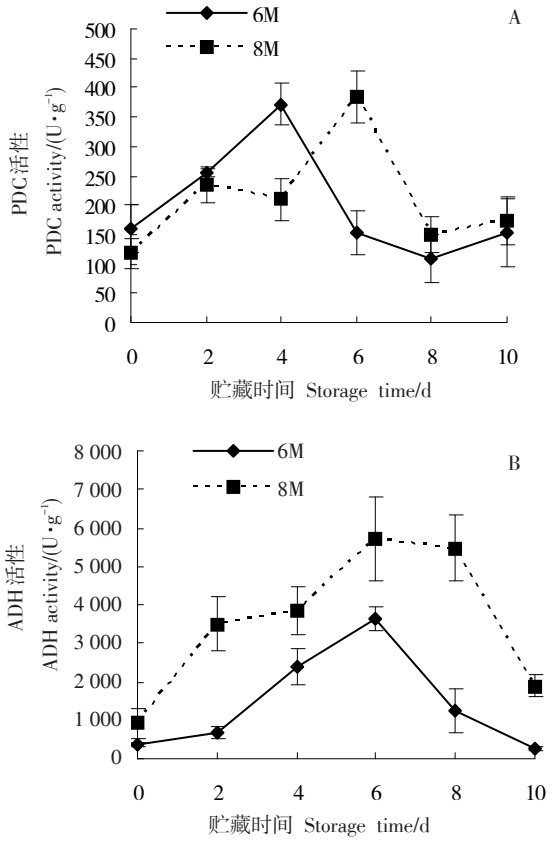


图3 果实在常温贮藏过程中PDC与ADH酶活性变化

Fig. 3 The changes of PDC and ADH activity in fruit during storage at 25 ℃

2.4 菠萝黑心病发病过程中PDC与ADH相对基因表达量变化

如图4-A所示，在菠萝果实常温贮藏过程中，6

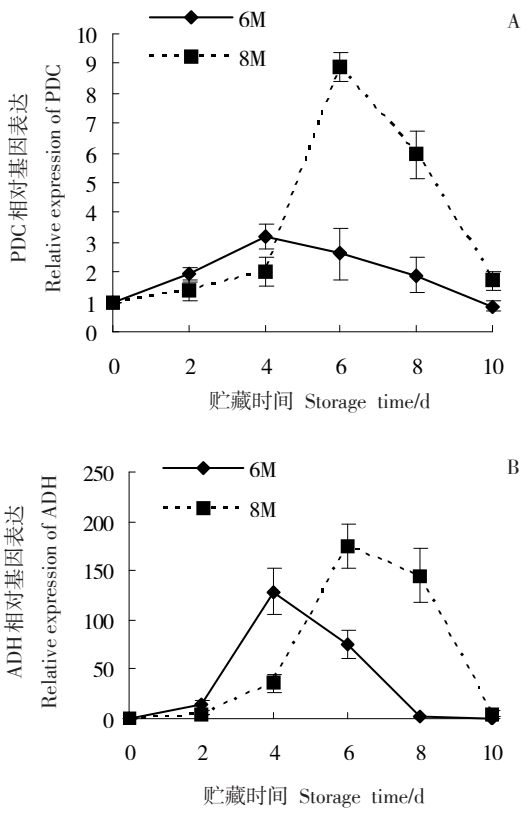


图4 果实在常温贮藏过程中PDC与ADH基因表达量变化

Fig. 4 The changes of PDC and ADH gene expression in fruit during storage at 25 ℃

M果PDC相对基因表达量在0~4 d时上升，4 d时到达峰值(3.19)，之后开始下降。8 M果PDC相对基因表达量同样先上升后下降，其峰值发生在6 d，为8.90。这2种成熟度果的PDC相对基因表达量变化均与其对应的丙酮酸脱羧酶活性变化一致。

如图4-B所示，在果实常温贮藏过程中，2种成熟度菠萝ADH相对基因表达量变化均呈先上升后下降趋势。6 M果ADH相对基因表达量在4 d时达到峰值，为128，显著低于8 M在6 d时达到的峰值(174)。并且这2种成熟度菠萝ADH相对基因表达量变化趋势与其对应的乙醇脱氢酶活性变化一致。

3 讨 论

采后果实褐变或黑心在很多水果贮藏过程中都会发生，如苹果[23]、梨[24]、李子[25]等。其影响因素主要包括贮藏温度、二氧化碳伤害、机械损伤等。梁丽雅等[24]研究发现果实成熟度是'鸭梨'果实褐变的主要因素，延迟采收更容易褐变，与本研究结果相反。本研究发现在室温贮藏条件下八成熟菠萝黑心病病情指数显著低于六成熟果，说明菠萝采收成熟度与采

后菠萝果实黑心病的发生密切相关。而‘巴厘’菠萝作为菠萝的主栽品种之一，其果实贮藏期间黑心病发病率超过90%，果农在生产中经常将成熟度较低的菠萝（如六成熟果）进行采收，可能是导致黑心病严重的原因之一。因此建议通过提高菠萝果实的采收成熟度来降低常温贮藏条件下菠萝黑心病发病率。

在果实采后贮藏过程中容易发生乙醇代谢过程[26]，在该过程中，乙醛、乙醇大量积累将导致果实品质劣变。丙酮酸的含量、PDC与ADH活性及其基因表达水平直接影响果实乙醇和乙醛的积累。因此，丙酮酸、乙醛和乙醇含量以及PDC与ADH活性及其基因表达水平高低是判断乙醇代谢快慢的重要指标[19,26-27]。李盼盼等[28]发现在常温贮藏条件下，猕猴桃果实丙酮酸含量呈先上升后下降趋势，而乙醇和乙醛含量急剧增加，并且PDC与ADH活性及其基因表达也相应上升。孙丽娜等[29]对冬枣贮藏过程中乙醇代谢及相关品质影响研究发现，室温贮藏期间冬枣果实中的丙酮酸、乙醇和乙醛含量均出现上升趋势，与本研究结果类似。本研究在菠萝贮藏中，伴随黑心病发生的过程，对其乙醇代谢途径相关指标进行了研究，表明6 M果和8 M果的PDC、ADH酶活性变化趋势一致，其中8 M果的PDC在贮藏前期上升速度高于6 M果，ADH酶活性在整个贮藏过程均为8 M果高于6 M果，该结果与其相对应的基因表达量变化趋势一致。但是采后菠萝果实黑心病的发生与乙醇代谢的相关性还有待于进一步的研究。

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