

新疆蜜脆苹果阿太菌果腐病菌 (*Athelia bombacina*)的分离与鉴定

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摘要:【目的】明确造成新疆维吾尔自治区博尔塔拉自治州双河市贮藏的蜜脆苹果出现褐色病斑的致病菌种类。【方法】选取具有典型症状的果实, 通过单孢分离法获得纯化菌株, 利用形态学特征并联合分子生物学, 明确菌株的分类地位, 基于科赫氏法则明确致病菌种类。【结果】为明确引起该病的病原菌, 对比形态学特征结合 ITS 和 LSU 基因片段联合分析后, 确定菌株 XJAU-PG-1 为阿太菌果腐病菌(*Athelia bombacina*)。【结论】研究首次报道了阿太菌果腐病菌能够侵染苹果果实。

关键词:蜜脆苹果; 致病性; 贮藏期病害; 阿太菌果腐病菌(*Athelia bombacina*)

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Isolation and identification of *Athelia bombacina* causing postharvest fruit rot of Honeycrisp apple in Xinjiang

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Abstract: 【Objective】The apple postharvest disease seriously affect the development of apple industry. In 2021, our research team found that dark brown spots occurred on the stored Honeycrisp apples in the cold storage of Shuanghe city, Xinjiang Uygur Autonomous Region (XUAR), and the fruit damage rate reached 50%. This study identified the pathogenic bacterium which would cause brown spots on Honeycrisp apples. 【Methods】The purified strains were obtained by single spore isolation, and were inoculated on apple fruits in vitro to identify the pathogenicity. The classification status of strains was determined by morphological characteristics and molecular technology. According to Koch's postulates, the fruits were inoculated through wound inoculation and no wound inoculation and were placed in a sterile fresh-keeping box. The inoculum was removed 24 h after inoculation and the lesion diameter was used to measure and record by the vernier caliper from the 3rd d to the 18th d. The strains were reisolated after the same lesions appeared, then we observed and recorded the morphology, color, growth rate and spore production of colonies on the second day. The mycelial diameter, spore morphology and size were observed and recorded under the optical microscope to clarify its morphological characteristics. The Ezup column fungal genome DNA extraction kit was used to extract DNA. The ITS1 and ITS4 were used to amplify the internal transcribed spacer (ITS) sequence, the NL1 and NL4 were used to amplify the ribosomal large subunit (LSU). The products were detected by 1% agarose gel electrophoresis. The primers and products were sequenced by Shanghai Sangon Bioengineering Co., Ltd (Shanghai, China).

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The sequences were spliced with seqman v.7.1.0 software and blast alignment. we constructed phylogenetic trees by Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian (BI) using combined polygenic fragments. The bootstrap support rate of MP and ML were greater than or equal to 50%, and BI was greater than or equal to 90%. Finally, the phylogenetic tree was used to clarify the taxonomic status of pathogens. 【Results】A total of 27 strains were isolated from the 45 diseased fruit tissue blocks with typical lesions. Among them, 14 strains were white colonies, and the highest isolation rate was 31.11%. There were 13 other strains, including 7 bacteria, 3 *Alternaria* fungi and 3 unknown fungi. The isolation rates of the strains with white colonies were 20%, 46.67% and 26.67% respectively when the disinfection time was 3, 5 and 7 min. After inoculating the strain into the fruit, the fruits of the control and no injury treatment had no disease. After 48 hours of treatment, yellowish brown micro round spots were formed. The hyphae penetrated and adhered to the fruit surface. When the culture time was extended to 15 days, the lesion color changed from yellowish brown in the initial stage to yellowish brown in the middle and dark brown at the edge. The average expansion rate of the diseased spots was $0.63 \text{ mm} \cdot \text{d}^{-1}$. The basidiospores and colonies basically consistent with the morphological characteristics of the original inoculated strain XJAU-PG-1 could be obtained from the inoculated diseased apple spots. Therefore, XJAU-PG-1 was further determined to be the pathogenic strain of apricot rot of Honeycrisp apple. The colony growth rate of strain XJAU-PG-1 was $15 \text{ mm} \cdot \text{d}^{-1}$ at $25 \text{ }^{\circ}\text{C}$ on PDA medium. The colony was always white and expanded radially and the initial hyphae were loose and thick. The basidiospores are oval or pear shaped, with slightly sharp and slightly skewed base, colorless and transparent, size $(2.4-3.6) \mu\text{m} \times (1.7-2.4) \mu\text{m}$. The phylogenetic analysis of polygenic sequences included 26 sequences, including 1590 total characters, 1135 constant characters, 82 variable characters and 373 parsimony-informative characters. The heuristic searched bootstrap method constructed an MP tree, $\text{TL} = 896$, $\text{CI} = 0.762$, $\text{RI} = 0.902$, $\text{RC} = 0.688$, $\text{HI} = 0.238$ for analysis. The ML tree and BI tree were consistent with the MP tree topology. The phylogenetic tree showed that strains XJAU-PG-1, XJAU-PG-2 and *A. bombacina* were clustered together with a support rate of 100/100/100. Combined with the morphological characteristics, strains XJAU-PG-1 and XJAU-PG-2 were identified as *Athelia bombacina*. 【Conclusion】Strain XJAU-PG-1 was isolated from the postharvest diseased Honeycrisp apple fruits. According to the morphological characteristics and the comprehensive analysis of molecular phylogeny of multi gene (ITS, LSU) fragments, strains XJAU-PG-1 and XJAU-PG-2 were identified as *A. bombacina*. Through the determination of its pathogenicity and the verification of Koch's rule, *A. bombacina* was determined to be the pathogen of apple fruit rot. This is the first time to report that *A. bombacina* was the pathogen of apricot rot occurred on Honeycrisp apple during storage.

Key words: Honeycrisp apple; Pathogenicity; Postharvest; *Athelia bombacina*

苹果是世界上主要的果树之一,苹果产业也是新疆林果产业的重要组成部分^[1]。蜜脆苹果具有产量高、品质优、适应性强等优点,市场前景十分广阔^[1-2]。而苹果采后病害的发生则降低了果实商品价值,给企业贮藏带来较严重的经济损失。

阿太菌果腐病是首次在中国河北省的黄冠梨上发生的一种贮藏期病害,病原菌为阿太菌果腐病菌(*Athelia bombacina*),危害症状为病斑浅褐色,圆形,病部可形成明显空腔且病健交界不明显,后期整

个果实表面布满白色菌丝,可闻到浓厚的菇香味^[3]。作者所在的研究组于2021年在新疆维吾尔自治区双河市冷库中发现贮藏的蜜脆苹果上出现黑褐色病斑,果实的受害率达50%,造成严重的经济损失,然而病原菌的种类尚不明确。笔者将具有典型病状的果实带回实验室,基于科赫氏法则明确致病菌种类,通过形态学结合分子生物学确定了致病菌的分类地位。研究结果不仅丰富了阿太菌果腐病的寄主种类,也为病害的准确诊断提供理论依据。

1 材料和方法

1.1 供试材料

供试材料来源于新疆维吾尔自治区双河市冷库中贮藏的蜜脆苹果果实。

培养基:马铃薯葡萄糖培养基(PDA)于121℃高压灭菌30 min后备用。

试剂:2×ES *Taq* Master Mix、4S Green Nucleic Acid、Ezup柱式真菌基因组DNA抽提试剂盒。

仪器:凝胶成像仪(WD-9413A)、PCR仪(Gray-96G)、电泳仪(DYY-12C)、电泳槽(DYCP-31DN)、超净工作台(SW-CJ-2D)、高压灭菌锅(LDZX-50KBS)、光照培养箱(SPX-300-GB)、光学显微镜(DN-107T)。

1.2 方法

1.2.1 菌株的分离、纯化 按照常规组织分离法,选取发病的病果,先用去离子水将果实表面冲洗干净,再用脱脂棉蘸取75%乙醇擦拭病斑部位,自然晾干后用无菌双面刀片切开果皮,取病健交界处约5 mm²大小的果肉,设置3、5、7 min 3个消毒时间,每个时间重复3皿,每皿5个组织块,共45个组织块。25℃黑暗培养3 d后纯化培养,待产孢后挑取单孢获得纯化培养菌株。将纯化菌株转接至PDA斜面上,置于4℃冰箱保存^[4-5]。

1.2.2 致病性测定 根据柯赫氏法则,选取大小一致、健康的蜜脆苹果10个,先将果实在自来水下清洗以去除表面灰尘,然后浸泡在1%的NaClO中消毒10 min,再用无菌水冲洗3次,自然晾干后备用^[6]。

采用有伤接种和无伤接种处理。有伤接种处理:在果实胴部位置,用5号昆虫针1针刺穿果皮,深度约5 mm,从培养4 d的菌落边缘打取直径5 mm的菌饼置于伤口上,然后用封口膜包裹,设置10个接种点,接种PDA培养基作为对照。无伤接种处理:在果实胴部位置随机选取接种点,设置10个接种点,接种PDA培养基作为对照^[6]。

将接种好的果实置于无菌保鲜盒中,培养温度25℃接种24 h后拆除接种体,第3天开始使用游标卡尺测量并记录病斑直径,至第18天。待出现相同病斑后参考1.2.1分离菌株。

1.2.3 菌株的形态学观察 将纯化后的菌株接种至PDA培养基上,隔天观察、记录菌落的形态、颜色、生长速度以及产孢情况。并在光学显微镜下观

察并记录菌丝直径、孢子形态及大小^[5]。

1.2.4 分子生物学研究 采用Ezup柱式真菌基因组DNA抽提试剂盒提取DNA^[5],选用引物ITS1(5'-TCCGTAGGTGAACCTGCGG-3')和ITS4(5'-TCCTCCGCTTATTGATATGC-3')扩增内转录间隔区(internal transcribed spacer, ITS)序列, NL1(5'-GCATATCAATAAGCGGAGGAAAAG-3')和NL4(5'-GGTCCGTGTTTCAAGACGG-3')扩增核糖体大亚基(large ribosomal subunit, LSU)序列^[5]。PCR反应体系均为30 μL,包含PCR master mix 15 μL,上下引物各1.5 μL, DNA模板1.5 μL, ddH₂O 10.5 μL。ITS引物PCR反应程序为:94℃预变性2 min, 94℃变性30 s, 51℃退火30 s, 72℃延伸40 s, 共35个循环,最后72℃延伸5 min; LSU引物PCR反应程序为:95℃预变性2 min, 95℃变性45 s, 55℃退火45 s, 72℃延伸1 min, 共35个循环,最后72℃延伸10 min。PCR产物用1%琼脂糖凝胶电泳检测,引物由生工生物工程(上海)股份有限公司提供,PCR产物测序由河南省京格商贸有限公司完成。

将获得的序列使用SeqMan v.7.1.0软件拼接后,通过BLAST比对搜索并下载同源性90%以上的基因序列进行比对,利用联合的多基因片段以最大简约法(MP)、最大似然法(ML)和贝叶斯法(BI)构建系统发育树。在MrBayes v. 3.1.2中使用马尔科夫链蒙特卡洛(MCMC)算法进行贝叶斯推理(BI)。使用1000个重复的bootstrapping(BS)方法评估MP和ML分析的分支支持。MP和ML的自举支持率≥50%、BI≥90%作为可信分支。系统图使用Figtree v. 1.3.1显示。用于系统发育分析序列信息见表1。

2 结果与分析

2.1 症状描述

蜜脆苹果果实在发病初期表面形成深褐色圆形病斑,后期病斑逐渐扩大,呈圆形或椭圆形(图1-A),病部有凹陷,切开病部果皮,可见病部果肉形成明显空腔。

2.2 菌株的分离

从具典型病斑的45个病果组织块中共分离得到27个菌株,其中菌落为白色的菌株共有14株,分离率最高,达31.11%;其他菌株13株,其中细菌7株、链格孢属真菌3株、未知真菌3株。分离率最高

表 1 系统发育分析中自测和 GenBank 下载的序列信息

Table 1 Sequences downloaded from GenBank and newly generated sequences obtained in phylogenetic analysis

种名 Species	菌株号 Isolate No.	寄主 Host	分布国家 Location	GenBank 登录号 Accession numbers		文献来源 Reference
				ITS	LSU	
<i>A. acrospora</i>	UC2022956	Litter or well decayed wood	USA	KP814375	—	[7]
<i>A. acrospora</i>	UC2022957	Litter or well decayed wood	USA	KP814332	—	[7]
<i>A. arachnoidea</i>	CBS 105.18	—	Germany	KY025592	MH866181	[8]
<i>A. arachnoidea</i>	CBS 418.72	<i>Populus</i> sp.	Netherlands	GU187504	GU187557	[9]
<i>A. bombacina</i>	HGAB	<i>Pyrus</i> spp.	China	MH201276	MH213145	[10]
<i>A. bombacina</i>	HXSAB	<i>Pyrus</i> spp.	China	MH201278	MH213147	[10]
<i>A. bombacina</i>	XLAB	<i>Pyrus</i> spp.	China	MH201277	MH213146	[10]
<i>A. bombacina</i>	UC2023122	On litter or well decayed wood in pinaceous forest	USA	KP814299	—	[7]
<i>A. bombacina</i>	XJAU-PG-1	<i>Malus pumila</i> Mill.	China	OK149651	OK157431	This study
<i>A. bombacina</i>	XJAU-PG-2	<i>Malus pumila</i> Mill.	China	OK560823	OK560851	This study
<i>A. decipiens</i>	GB0090493	—	Sweden	LR694193	LR694170	[11]
<i>A. decipiens</i>	TUFC 14532	Culture	Japan	LC516617	—	[12]
<i>A. decipiens</i>	L-10567	—	USA	GU187537	GU187592	[9]
<i>A. epiphylla</i>	TUFC 33567	Culture	Japan	LC516618	—	[12]
<i>A. epiphylla</i>	TUFC 33568	Culture	Japan	LC516619	—	[12]
<i>A. neuhoffii</i>	GB0087199	—	Sweden	LR694195	LR694172	[11]
<i>A. pellicularis</i>	CCFC 30532	—	USA	U85799	—	[13]
<i>A. pyriformis</i>	Hjm 18581 (GB)	—	Sweden	EU118605	—	[13]
<i>A. rolfsii</i>	ATCC 201126	—	—	AF499018	AF499019	[14]
<i>A. rolfsii</i>	SPL15006	<i>Ipomoea batatas</i>	Korea	KY446390	KY446370	[15]
<i>A. rolfsii</i>	SPL15004	<i>Ipomoea batatas</i>	Korea	KY446392	KY446370	[15]
<i>A. singularis</i>	JS 25630 (GB)	—	Norway	GQ162813	GQ162813	[15]
<i>A. termitophila</i>	TUFC 14530	Culture	Japan	LC516620	LC516626	[12]
<i>A. termitophila</i>	TUFC 31133	Culture	Japan	LC516621	LC516627	[12]
<i>A. termitophila</i>	TUFC 34057	Culture	Japan	LC516622	LC516628	[12]
<i>A. termitophila</i>	TUFC 34079	Culture	Japan	LC516623	LC516630	[12]
<i>Granulobasidium vellereum</i>	CBS 124595	—	Netherlands	MH863395	MH874913	[11]

注: XJAU-PG-1 和 XJAU-PG-2 为本研究菌株;—为无相关序列。

Note: Strain of this study are XJAU-PG-1 and XJAU-PG-2; —. Not applicable.

的菌株在消毒时间为 3、5、7 min 时的分离率分别为 20%、46.67% 和 26.67%。

2.3 致病性测定

将菌株接种至果实后,对照和无伤处理均未发病(图 1-B)。有伤处理 48 h 后形成肉眼可见的黄褐色微圆病斑,接种 15 d 后,病斑由初期的黄褐色转为中部黄褐、边缘黑褐色轮纹状(图 1-C~D);病斑的平均扩展速率为 0.63 mm·d⁻¹。从接种发病的苹果病斑上刮取少量的病组织镜检,单孢分离培养,可获得与接种菌株 XJAU-PG-1 形态特征基本一致的孢子形态,确定 XJAU-PG-1 为蜜脆苹果阿太菌果腐病的致病菌株。

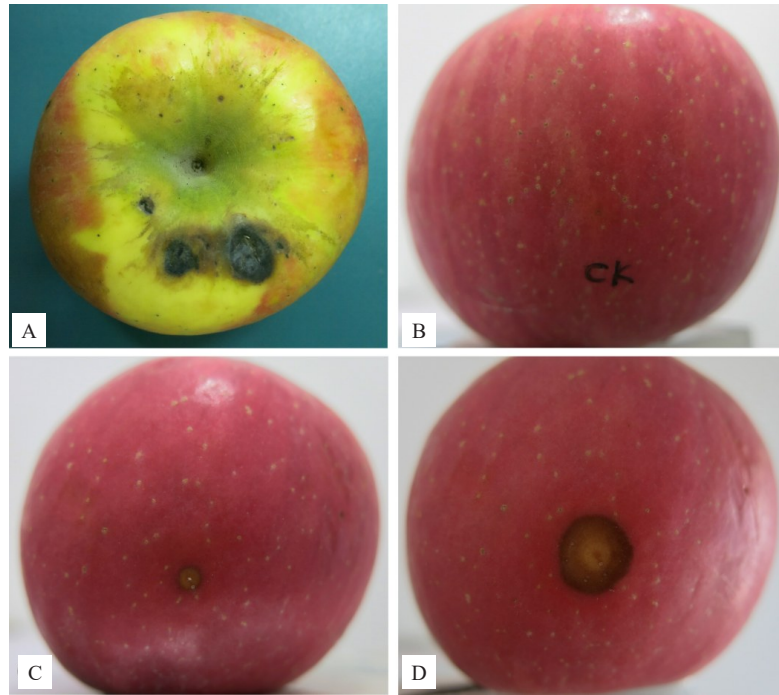
2.4 菌株的形态学鉴定

由图 2 可知,菌株 XJAU-PG-1 在 25 °C 条件下菌

落生长速率为 15 mm·d⁻¹,菌落始终为白色,呈辐射状扩展(图 2-B);菌丝初较疏松,密集成层;担孢子椭圆形或梨形,基部略尖且稍歪斜,无色透明(图 2-D),大小为(2.4~3.6)μm×(1.7~2.4)μm。

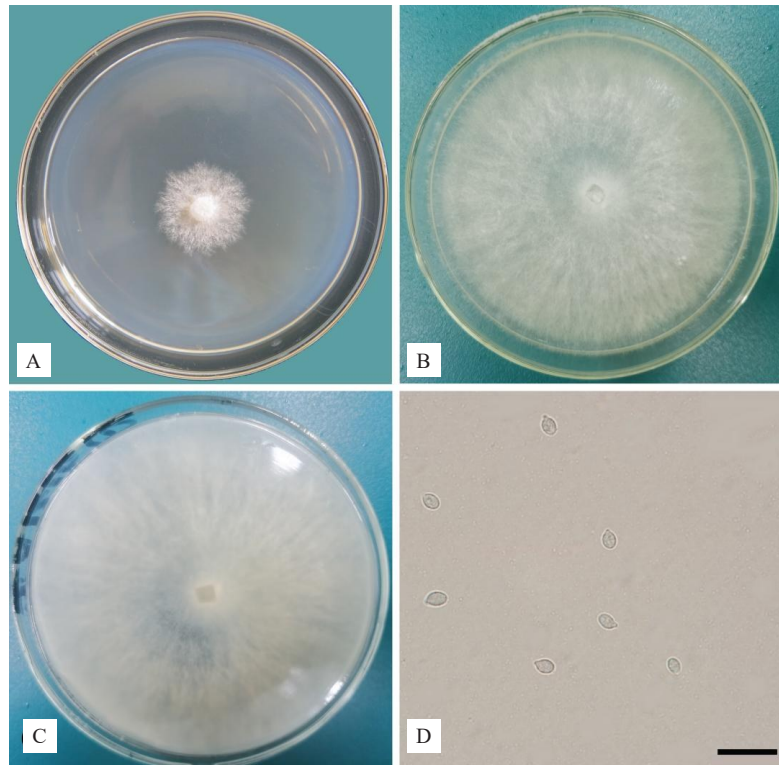
2.5 菌株的分子鉴定

PCR 分别扩增基因组 DNA 的 ITS 和 LSU 片段,经 BLAST 比对分析后,从 GenBank 数据库中下载与菌株相似度达 90% 以上的 ITS、LSU 序列,以 *Granulobasidium vellereum* 为外群,联合构建系统发育树。多基因序列的系统发育分析共包括 26 个序列,其中共有 1590 对碱基位点,包括 1135 个连续性碱基位点,82 个可变碱基位点,373 个简约信息位点。采用启发式搜索 Bootstrap 方法构建 1 个 MP 树,以 TL=



A. 阿太菌果腐病侵染蜜脆苹果自然发病症状; B. 果实对照; C. 接种 4 d 后果实发病症状; D. 接种 15 d 后果实发病症状。
 A. The natural symptom of *Athelia bombacina* causing postharvest fruit rot on Honeycrisp apple; B. Fruit control; C. Symptoms occurred 4 days after fruit inoculation; D. Symptoms occurred 15 days after fruit inoculation.

图 1 病原菌致病性测定
 Fig. 1 Pathogenicity test



A. 培养 3 d(正面); B. 培养 9 d(正面); C. 培养 9 d(反面) D. 担孢子; 标尺=10 μm.
 A. Culture for 3 d (front); B. Culture for 9 d (front); C. Culture for 9 d (back); D. Basidiospore; Scale bars=10 μm.

图 2 菌株 XJAU-PG-1 在 PDA 培养基上的菌落形态
 Fig. 2 Colony morphology of strain XJAU-PG-1 on PDA medium

896、CI=0.762、RI=0.902、RC=0.688、HI=0.238 进行分析。ML 树和 BI 树与 MP 树拓扑结构一致。结果显示菌株 XJAU-PG-1、XJAU-PG-2 与 *A. bombacina*

以 100/100/100 的支持率聚类在同一分支(图 3), 结合该菌株形态学特征确定菌株 XJAU-PG-1 与 XJAU-PG-2 为 *Athelia bombacina*。

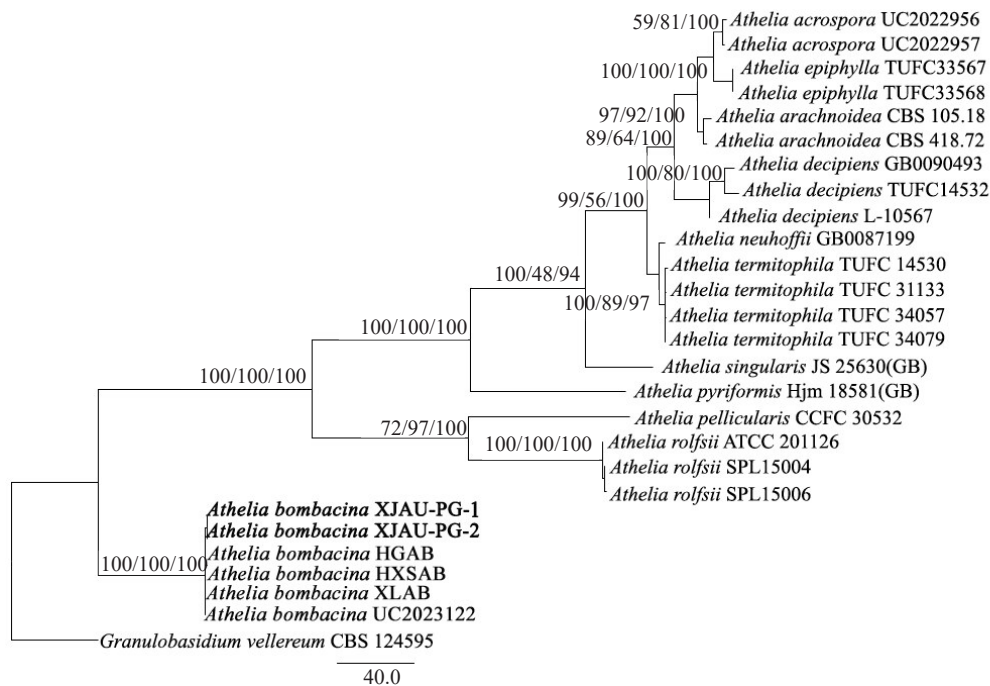


图 3 基于 MP 的多基因片段(ITS、LSU)系统发育树(MP/ML/BI)

Fig. 3 Phylogrm of combined ITS and LSU gene generated from maximum parsimony (MP) analyses

3 讨 论

阿太菌属(*Athelia*)真菌隶属于担子菌门(Basidiomycota), 伞菌纲(Agaricomycetes), 伞菌亚纲(Agaricomycetidae), 阿太菌目(Atheliales), 阿太菌科(Atheliaceae)。该属真菌中仅对能够危害 200 多种植物引起白绢病的罗耳阿太菌(*A. rolfsii*)研究较多^[16-17]。阿太菌果腐病菌(*Athelia bombacina*)目前仅在北非的桉树属(*Eucalyptus* sp.)、南非的赤松(*Pinus densiflora*)、德国的欧洲云杉(*Picea abies*)以及美国的苹果(*Malus pumila*)落叶、黑云杉(*Picea mariana*)、北美云杉(*Picea sitchensis*)、扭叶松(*Pinus contorta* var. *contorta*)、欧洲山杨(*Populus tremuloides*)、甘比耳氏栎(*Quercus gambelii*)上有记载^[18-20], 该菌在前期研究中主要作为苹果黑星病(*Venturia inaequalis*)和苹果斑幕潜叶蛾的生防菌株被报道^[21-23]。贾晓辉^[3]于 2019 年的研究表明 *A. bombacina* 表现出对不同病原菌较强的抑菌能力。

A. bombacina 作为致病菌的报道较少。2012 年, Toda 等^[24]在贮藏的甜菜根中分离到与 *A. bom-*

bacina 亲缘相近的致病菌; Jia 等^[10]于 2015 年首次在中国农业科学院果树研究所贮藏的库尔勒香梨上发现了阿太菌果腐病 [*Athelia bombacina* (Link) Pers.]; 2016—2018 年, 河北地区贮藏期的黄冠梨中发现由 *A. bombacina* 引起的阿太菌果腐病, 发病率高达 10%~20%, 对企业造成较严重的经济损失^[3-4]; 目前, 阿太菌果腐病仅在库尔勒香梨、黄冠和红香酥等梨品种上有记录^[3-4]。国外仅 Andrews 在美国的苹果 (*Malus pumila*) 落叶中收集到该菌^[3], 笔者首次在新疆维吾尔自治区的双河市发现 *A. bombacina* 可引起苹果贮藏期病害。

Heye^[18] 研究发现 *A. bombacina* 与许多担子菌不同。其在培养基上生长迅速, 也可以在液体培养中生长; 温度范围很广, 在 4 °C 下可存活 18 个月之久, 低温以及高湿环境更有利于其生长^[13, 24]。而大多果品贮藏库湿度高且温度大多在 0~4 °C 之间^[25-26], 为病原菌的生长提供条件。本研究中, 菌株 XJAU-PG-1 在室温条件(25 °C)下培养生长迅速, 且蜜脆苹果在低温高湿黑暗条件的冷库中贮藏。因此, 该病害至今仅在贮藏环境下发现。前期并未

发现该菌侵染苹果,阿太菌果腐病是否由采前潜伏侵染所致,对采前苹果枝干、叶、花、幼果等是否具有致病力都尚未明确^[3]。

贾晓辉等^[4,25]的室内寄主范围测定结果表明,*A. bombacina*除可侵染梨和苹果外,还可侵染樱桃、桃、杏和枣等多种核果类果树,将其作为苹果黑星病的生防菌是否具有风险有待进一步研究。

4 结 论

从受害蜜脆苹果果实上分离获得菌株 XJAU-PG-1,通过柯赫氏法则验证,明确其为苹果阿太菌果腐病的病原菌。依据形态学特征结合多基因(ITS、LSU)片段分子系统发育综合分析后,首次明确了危害贮藏期蜜脆苹果引起阿太菌果腐病的病原菌为阿太菌果腐病菌(*Athelia bombacina*)。

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