果 树 学 报 2022,39(4):644-651
Journal of Fruit Science

DOI:10.13925/j.cnki.gsxb.20210461

来自台湾地区番荔枝果实上可可花瘿病菌的检疫鉴定

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摘 要:【目的】从台湾进境的番荔枝果实样品上发现带有黑褐色、不规则病斑的病果,对其进行病原菌分离鉴定,以明确其病原种类及分类地位。**【方法】**采用常规PDA平板分离法获得1株疑似可可花瘿病菌(*Albonectria rigidiuscula*)菌株2268-2-2-3,通过形态学特征观察、序列比对分析及致病性测定的方法对其进行了鉴定。**【结果】**分离菌株在PDA平板上菌丝生长茂盛,菌落呈粉色,可产生大量大型分生孢子和小型分生孢子,大型分生孢子镰刀形或稍弯的柱形,具5~9个隔,大小为(52.98~76.33)μm×(5.38~8.56)μm,小型分生孢子椭圆形至圆柱形,有0~1个隔,大小(4.95~10.38)μm×(2.61~5.12)μm,培养期间未见有性阶段。经rDNA基因间隔序列(ITS)和翻译延长因子基因序列(EF1A)比对分析,发现该菌株与GenBank中多个可可花瘿病菌(*A. rigidiuscula*)菌株的ITS和EF1A基因序列同源性超过98%。根据EF1A序列构建的系统进化树表明,该菌株与其他多株可可花瘿病菌(*A. rigidiuscula*)分离物EF1A序列聚在同一个分枝上。致病性测定结果表明菌株2268-2-2-3可侵染释迦果实,形成黑褐色不规则病斑。以上研究结果表明台湾地区释迦果上分离获得的菌株2268-2-2-3为可可花瘿病菌(*A. rigidiuscula*)。**【结论】**采用形态学、序列分析、致病性测定相结合的方法,准确截获鉴定了来自台湾地区释迦果上的可可花瘿病菌。可可花瘿病菌是我国进境检疫性植物病原真菌,研究为口岸检疫以及该病菌的防控工作提供了重要参考。

关键词:番荔枝;果实;可可花瘿病菌;检测鉴定

中图分类号:S667.9 **文献标志码:**A **文章编号:**1009-9980(2022)04-0644-08

Quarantine identification of *Albonectria rigidiuscula* on the custard apple from Taiwan of China

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Abstract:【Objective】Custard apple (*Annona squamosa* L.) is an edible tropical fruit, and is also called sugar apple or sweetsop. Custard apple is a very delicious fruit, valued for the flavor and texture of its pulp. Fruit also has high caloric value and sugar content. Owing to domestic market demand, China has expanded its imports. According to customs statistics, the domestic import of custard apple was 135 000, 118 000, 80 000, 132 000 and 153 000 tons from 2015 to 2019. China imported 124 000 tons of custard apple in 2020, 95% of which was produced from Taiwan province. To import the custard apple, it is required to apply from the plant quarantine authority for issuance of quarantine requirements and shall be in compliance with the quarantine requirements. Symptoms of the black irregular spots were observed in Fuzhou port. The experiment was undertaken to identify and characterize the pathogen that caused black brown spot of custard apple.【Methods】Strain 2268-2-2-3 was isolated from custard apple imported from Taiwan of China by using conventional PDA plate method. To identify strain 2268-2-2-3, the morphological characteristics, sequence alignment and its pathogenicity were analyzed. Fungal isolate was incubated on PDA for 14 days in the dark to observe the morphological characteris-

收稿日期:2021-09-29 **接受日期:**2021-12-23

基金项目:国家重点研发计划(2016YFF0203201&2016YFF0203203);国家自然科学基金(NSFC31770009);海关总署科研项目(2020HK162);福州海关科研项目(FK2021-02)

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tics, after which 40 mg fresh mycelia were collected and used for DNA extraction. The mycelia were homogenized in the tube using the mixer mill MM 400 for 90 seconds at the frequency of 30 times per second. Genomic DNA extraction was conducted using Labserv Plant DNA Extraction kit with a KingFisher mL machine, according to the instructions of the manufacturer. DNA concentrations were determined using a Nanodrop ND-1000 spectrometer. Then the DNA solutions were used as the templates for PCR. The internal transcribed spacer (ITS) of nuclear rDNA and translation elongation factor 1-alpha (EF1A) regions of the strain 2268-2-2-3 were amplified in PCR assays using universal primers ITS1/ITS4 and EF1-728F/EF1-986R. Nuclear ribosomal DNA (rDNA) region is relatively conserved within the species but variable among species, and thus is a useful region for distinguishing different species. Based on rDNA sequences, some diagnosis protocols have been successfully developed for quarantine fungi. However, in our experiment, we found it was difficult to identify the *A. rigidiuscula* by ITS sequence. Then, EF1A sequences belonging to the *A. rigidiuscula* and related species were further used to generate phylogenetic trees with the neighbor joining (NJ) methods by using the MEGA 6.0. The phylograms were bootstrapped 1000 times to assess the degree of support for the pylogenetic branching indicated by the optimal trees. Trees were rooted with *Fusarium circinatum* (MW402083, MW402082) and *Fusarium begoniae* (MW401969, MW401968) as outgroup. To fulfill Koch's postulates, strain 2268-2-2-3 was tested for pathogenicity to fruit of custard apple by artificial inoculation. The method included wounding of the fruits by sterile knife, inoculating into wounded fruits by placing agar of mycelium and obtaining the sample from 10-day-old culture plates. The control was used by inoculating the wounded fruits with PDA agar. Inoculated fruits and controls were covered with sterile cotton wool and placed in the polyethylene bags. Each inoculated fruit was placed in a moist chamber and incubated at 25 ℃. Four fruits were used per treatment.【Results】The strain 2268-2-2-3 grew well on PDA medium. The mycelium was pink and could produce a large number of macroconidia and microconidia. Macroconidia were sickle-shaped or slightly curved columnar, with 5-9 septa, 52.98-76.33 μm long, and 5.38-8.56 μm wide. Microconidia were elliptic to cylindrical, with 0-1 septa, 4.95-10.38 μm long and 2.61-5.12 μm wide. There was no sexual stage during the cultural period. The ITS and EF1A sequences of strain 2268-2-2-3 was compared with those in GenBank database, showing that the sequence shared more than 98% homology with several sequences of *A. rigidiuscula* in GenBank, respectively. The phylogenetic tree based on EF1A sequences showed that the strain 2268-2-2-3 was gathered with other *A. rigidiuscula* isolates in a branch. The strain 2268-2-2-3 could infect sirikaya, forming dark brown irregular spots in the pathogenicity test.【Conclusion】*A. rigidiuscula* is an aggressive pathogen causing gall disease of cocoa and is listed as a quarantine fungus in China. However, identification and quarantine diagnosis of *A. rigidiuscula* based only on morphology is problematic due to its morphological plasticity and delayed appearance of the fruiting bodies. Combined with the morphological characteristics, by using ITS and EF1A sequence analysis, the fungi were identified as *A. rigidiuscula*, which can cause dark brown irregular spots on custard apple. To our knowledge, this is the first report of interception of *A. rigidiuscula* on custard apple in China, providing great values for quarantine practice in the ports.

Key words: Custard apple; Fruit; *Albonectria rigidiuscula*; Detection and identification

番荔枝是番荔枝科（Annonaceae）番荔枝属（*Annona*）植物[1]，为世界五大热带水果之一，其果肉鲜嫩、营养丰富，芳香清甜，深受消费者喜爱[2]。番荔枝原产热带美洲，现亚洲热带地区也有种植，我国台湾、广东、广西、海南、福建、云南、贵州等省（区）均有分布，其中台湾地区种植最多[1, 3-4]。目前，我国番荔枝果实只能从台湾地区和泰国进口，根据海关数据统计，2015—2019年，国内番荔枝果实进口量分别

是1.35万、1.18万、0.8万、1.32万和1.53万t，呈现出上下波动的态势；2020年全国进口番荔枝果实1.24万t，其中95%来自台湾地区。番荔枝果实在生长、采收、贮藏及运输过程中极易受多种病原菌的侵染，特别是当其受到机械损伤果皮破损时，病原菌可通过破损处进入果实繁殖，导致果实病变、腐烂[5-6]。有研究表明引起番荔枝果实病害的种类与地区有关，如戚佩坤等[7]调查发现广东省番荔枝果实病害最主要的病原菌是黑腐病菌（*Botryodiplodia theobromae*）、类叶点霉叶斑病菌（*Phyllostictina anonicola*）、根腐病菌（*Cylindrocladiella tenuis*）及围小丛壳（*Glomerella cingulata*），而台湾地区番荔枝果实的病害主要是由拟茎点霉（*Phomopsis* sp.）、镰孢菌（*Fusarium* sp.）、炭疽病菌（*Colletotrichum* sp.）、疫霉菌（*Phytophthora citrophthora*）、黑腐病菌（*Botryodiplodia theobromae*）等病原菌引起的[3,8]。

可可花瘿病菌[*Albonectria rigidiuscula*（Berk. & Broome）Rossman & Samuels]属真菌界（Fungi）、子囊菌门（Ascomycota）、粪壳菌纲（Sordariomycetes）、肉座菌目（Hypocreales）、丛赤壳科（Nectriaceae）、白壳属（*Albonectria*），曾用名*Nectria rigidiuscula* Berk. & Broome，其无性阶段也被称之为多隔镰刀菌（*Fusarium decemcellulare* Brick）[9]，危害可可（*Theobroma cacao*）、杧果（*Mangifera indica*）、毛叶枣（*Ziziphus mauritiana*）、榴莲（*Durio zibethinus*）、玉米（*Zea mays*）、豆科（Leguminosae）、罗汉松（*Podocarpus macrophyllus*）等多种植物[10-17]。该病菌广泛分布于亚洲、非洲、北美洲、大洋洲、南美洲等多个国家[10-16]，目前，在我国台湾、四川、湖北等地有局部分布[15-16,18]。该菌现已被列入我国进境植物检疫性有害生物名录，是一种检疫性植物病原真菌[19]。

2021年4月，福州海关技术中心从来自台湾的番荔枝果实上分离获得了1株疑似可可花瘿病菌（*A. rigidiuscula*）菌株，通过形态学特征观察、致病性测定和序列比对分析，对其进行了检疫鉴定。

1 材料和方法

1.1 材料、试剂和仪器

1.1.1 *样品来源和供试菌株* 采用常规平板分离法从台湾进境的番荔枝果实样品上分离获得1株疑似可可花瘿病菌（*A. rigidiuscula*）菌株，经单孢分离后得到纯化菌株2268-2-2-3。

1.1.2 *培养基* 马铃薯葡萄糖琼脂（PDA）培养基[20]。

1.1.3 *试剂* TANbead® Plant DNA Auto Kit试剂盒（台湾奈米技术开发有限公司）；*Taq* DNA聚合酶、$MgCl_2$、10×PCR Buffer、DL2000 Marker、DNA片段纯化试剂盒[宝生物工程（大连）有限公司]。

1.1.4 *仪器* Imager Z1显微镜数码成像系统、Discovery V12解剖镜及其成像系统（德国Zeiss公司）；Friocell 222型生化培养箱（德国3M公司）；Kingfisher mL型核酸自动化提取仪、NanoDrop 2000C型超微量分光光度计（美国Thermo Fisher公司）；TProfessional Basic PCR仪（德国Biometra公司）；PowerPac Basic电泳设备、GelDocEQ型凝胶成像系统（美国Bio-Rad公司）。

1.2 方法

1.2.1 *菌株的形态学特征观察* 将供试菌株2268-2-2-3接种于直径9 mm的PDA平板上，于25 ℃下连续暗培养14 d后，观察记录菌落正反面的颜色和气生菌丝的疏密程度。用解剖针自PDA平板上挑取菌落或者菌落中央黏孢团，制片后在显微镜下观察大、小分生孢子的形态特征，随机各挑取30个大型分生孢子和小型分生孢子进行形态测量。

1.2.2 *基因组DNA提取* 用灭菌枪头刮取PDA平板上培养14 d的菌株2268-2-2-3菌丝体40 mg，按照TANbead® Plant DNA Auto Kit试剂盒使用说明书，在核酸自动化提取仪上进行基因组DNA提取[17]。提取后的DNA经超微量分光光度计检测浓度后，冻存于−20 ℃冰箱备用。

1.2.3 *序列扩增与测定* 利用所提取菌株2268-2-2-3的基因组DNA，采用引物ITS1（5'-TCCGTAGGTGAACCTGCGG-3'）/ITS4（5'-TCCTCCGCTTATTGATATGC-3'）扩增rDNA基因间隔序列（ITS）片段[21]、引物EF1-728F（5'-CATCGAGAAGTTCGAGAAGG-3'）/EF1-986R（5'-TACTTGAAGGAACCCTTACC-3'）扩增翻译延长因子（translation elongation factor 1-alpha，EF1A）基因片段[22]。PCR扩增产物经2%琼脂糖凝胶电泳检测，用凝胶成像系统进行成像分析，扩增成功后按照DNA片段纯化试剂盒说明书进行纯化。纯化产物送上海华大基因科技有限公司进行双向测序。

1.2.4 *序列比对分析* 利用NCBI网站上的BLAST程序对所得的ITS和EF1A序列进行比对分析，确认

序列的可靠性。使用MEGA 6.0软件对测得的菌株2268-2-2-3的EF1A序列与GenBank上下载的可可花瘿病菌（*A. rigidiuscula*）及其相近序列进行比对，以*Fusarium circinatum*（MW402083、MW402082）和*Fusarium begoniae*（MW401969、MW401968）的EF1A序列为外群，采用邻接法（neighbor-joining method，NJ）构建系统进化发育树，进行1000次重抽样计算系统树中节点的置信度。

1.2.5　致病性测定　选用健康番荔枝果实进行接种试验。用灭菌刀在番荔枝果实上切4 mm小口，挑取在PDA平板上培养10 d的菌丝块，将菌丝块接种到番荔枝果实的切口上，用湿润的无菌脱脂棉覆盖，放入封口袋中，置于25 ℃培养箱中培养，定期观察记录发病情况。接种4个番荔枝果实，另外用无菌PDA琼脂块接种3个番荔枝果实实作为对照。

2　结果与分析

2.1　症状观察

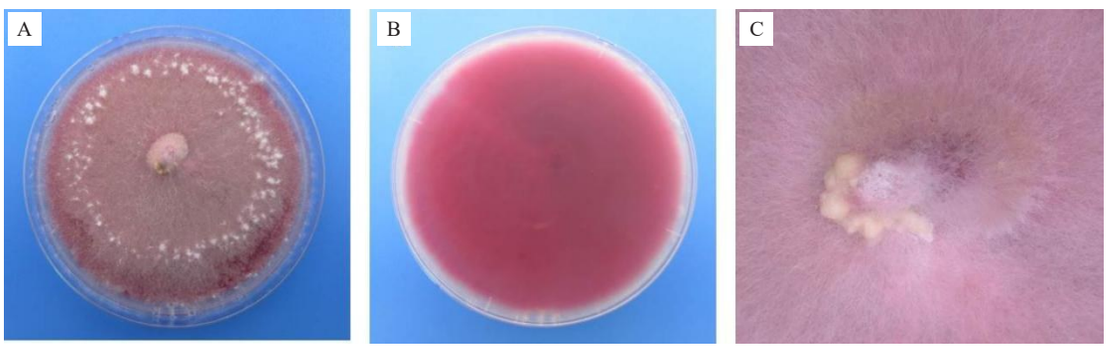
送检番荔枝果实的果蒂附近有密集小而浅的黑色不规则点状病斑（图1），后期逐渐连成一片形成黑褐色不规则的大病斑。

图1　番荔枝果实上黑色不规则病斑

Fig. 1　Symptoms of the black irregular spots on custard apple fruit

2.2　菌落性状

在PDA培养基上，菌丝生长茂盛，培养皿上菌落呈粉红色，底部红色。培养基中央可产生乳黄色黏孢团，内有大量大型分生孢子（图2）。



A. 菌落正面；B. 菌落背面；C. 乳黄色黏孢团。

A. Colony front view; B. Colony reverse view; C. Creamy yellow sporodochia.

图2　菌株2268-2-2-3在PDA上培养菌落性状（培养14 d）

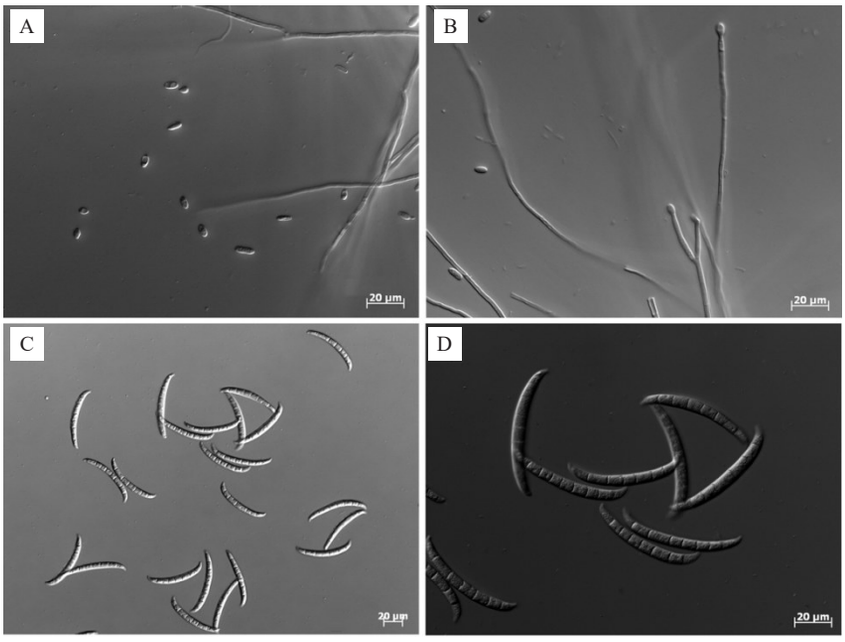
Fig. 2　Culture characterization of strain 2268-2-2-3 after 14 days incubation on PDA mediu

2.3　病菌形态特征

菌株2268-2-2-3在PDA培养基上可产生小型分生孢子和大型分生孢子。小型分生孢子着生于气生菌丝的小梗上，串生、假头生，分生孢子梗无色，小型分生孢子椭圆形至圆柱形，无色透明，具0~1个隔，大小（4.95~10.38）μm×（2.61~5.12）μm（图3-A、B）。大型分生孢子非常大，镰刀形或稍弯的柱形，壁厚，孢子顶端略弯曲并缩小，多孢，有5~9个隔，大小为（52.98~76.33）μm×（5.38~8.56）μm（图3-C、D）。未观察到病原菌的有性态。

2.4　序列比对分析

菌株2268-2-2-3 DNA分别利用真菌通用引物ITS1/ITS4、EF1-728F/EF1-986R扩增后获得的片段长度约为528、309 bp。在GenBank中进行Blastn分析，结果表明菌株2268-2-2-3所测ITS序列与GenBank中登录号为KX788159、KM893858等可可花瘿病菌（*A. rigidiuscula*）的ITS序列同源性达99%以上。EF1A序列与GenBank中登录号为MG857267、LC081241等可可花瘿病菌（*A. rigidiuscula*）的EF1A序列同源性超过98%。



A. 小型分生孢子；B. 分生孢子梗；C 和 D. 大型分生孢子。

A. Microconidia; B. Conidiophores; C & D. Macroconidia.

图 3 菌株 2268-2-2-3 在 PDA 上形态特征（培养 14 d）

Fig. 3 Morphological characterization of strain 2268-2-2-3 after 14 days incubation on PDA medium

系统发育分析结果显示，菌株2268-2-2-3的EF1A序列与GenBank中下载的可可花瘿病菌（*A. rigidiuscula*）EF1A序列聚在同一个分枝，Bootstrap支持值为99（图4）。

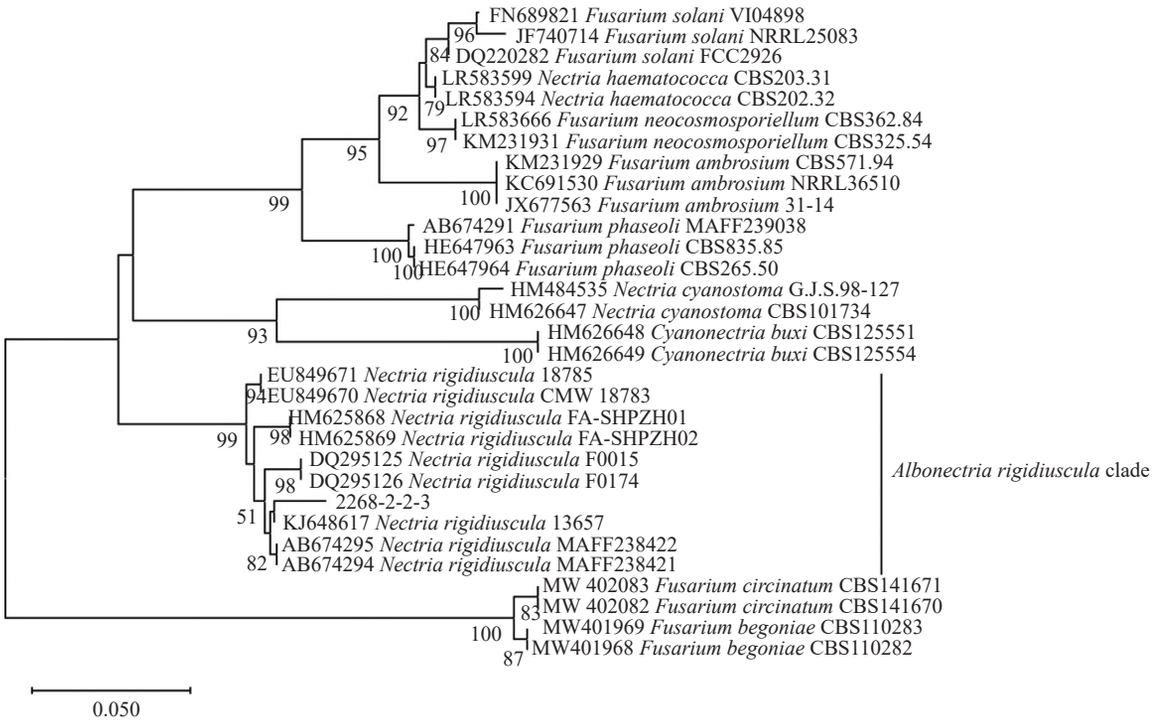
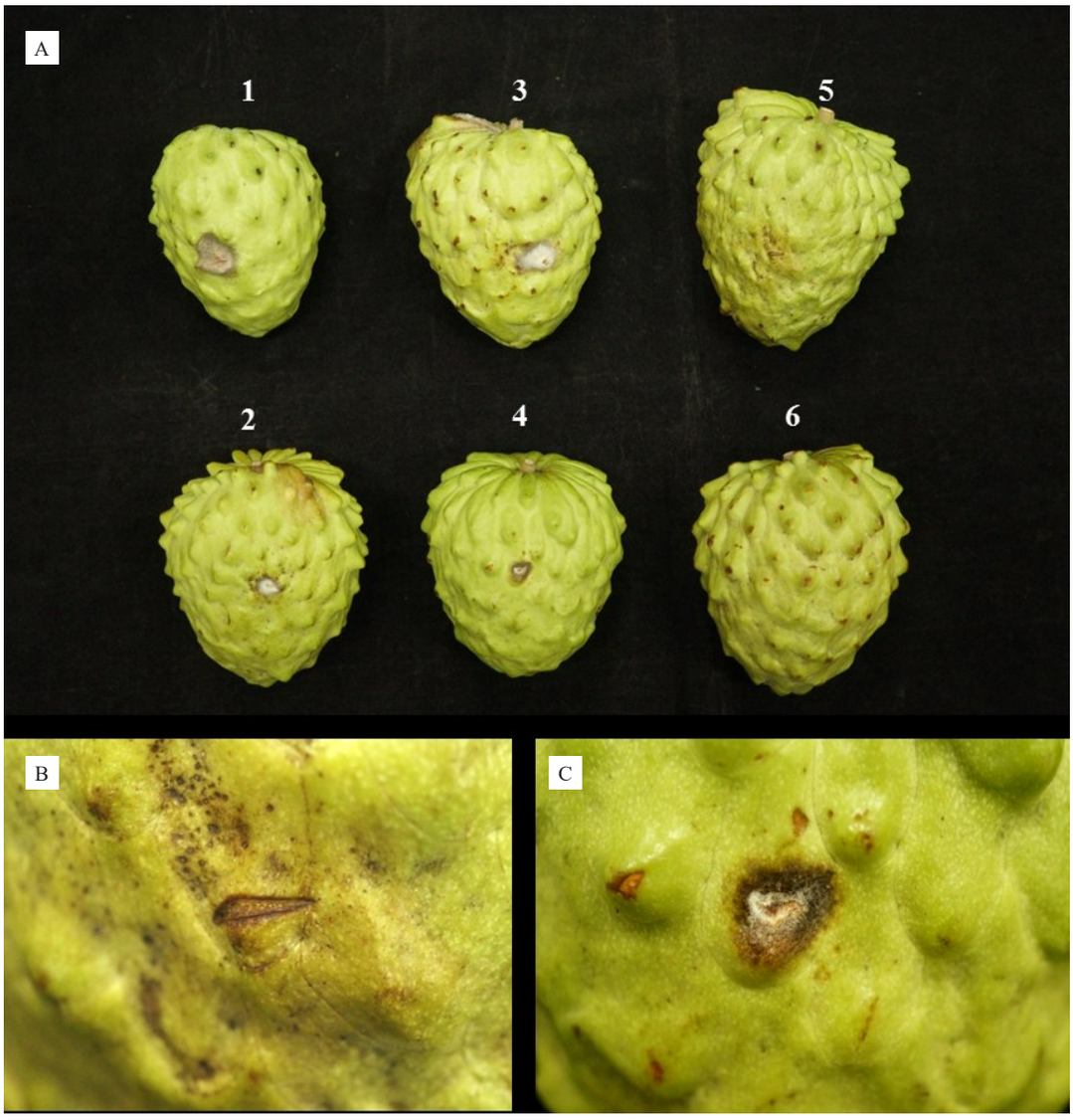


图 4 基于菌株 2268-2-2-3 及其相近种的 EF1A 序列采用邻接法构建的系统发育树

Fig. 4 Phylogenetic tree based on the Neighbor-joining (NJ) ananlysis inferred from the translation elongation factor regions of 2268-2-2-3 and the related species

2.5　致病性测定

菌株2268-2-2-3接种7 d后观察番荔枝果实发病情况（图5），在接种点附近出现黑褐色不规则病斑，与原症状一致。对病斑进行分离获得与原病菌形态、分子序列一致的菌株。无菌水对照组未出现发病症状。



A. 接种 7 d 后症状（1~4. 接种组，5~6. 对照组）；B. 对照组；C. 接种组。

A. Inoculation symptoms after 7 days (1~4. Inoculated group, 5~6. Control group); B. Control group; C. Inoculated group.

图 5　接种后症状

Fig. 5　Inoculation symptoms

3　讨　论

在可可花瘿病菌的检疫中，菌落形态和颜色、大型分生孢子形态和大小是鉴定该病菌的重要指标。在PDA培养基上，可可花瘿病菌菌丝生长较慢，菌落开始为白色，后逐渐变为乳白色至粉色或黄色，大型分生孢子大小为（68.5~97.5）×（7.5~9.2）μm，呈镰刀形或稍弯的柱形，通常有5~9个隔[10]。本研究中，菌株2268-2-2-3在PDA上经过培养后，菌落呈粉红色，有大量大型分生孢子产生，其形态和大小与SN/T 3284—2012标准及以往研究报道[9,17]中的描述基本一致。

可可花瘿病菌可引起枝条溃疡、花序枯萎及维管束坏死，该病害在国外报道发生较多，如在巴西有该病菌危害腰果树（*Anacardium occidentale*）、咖啡属（*Coffea canephora*）植物的报道[23-24]；在委内瑞拉、古巴有该病菌危害可可树（*Theobroma cacao*）的报道[12,25]；在越南有该病菌危害越南黄檀（*Dalbergia*

tonkinensis）的报道[26]；在多米尼加共和国、波多黎各有该病菌危害杧果树（*Mangifera indica*）的报道[27-28]，我国四川省攀枝花市有该病菌造成杧果树枯死的报道[15]。Togawa等[11]研究表明可可花瘿病菌可危害番荔枝树，该病菌在番荔枝树上发病症状主要为新梢顶枯。Yunianto等[29]从斯里兰卡的番荔枝上分离到4种内生真菌，其中一种为可可花瘿病菌（*A. rigidiuscula*）。目前，关于该病菌对番荔枝果实的危害症状还未有详细报道。而本文采用形态学特征、序列分析及致病性测定的方法，准确鉴定了来自台湾番荔枝果实上的疑似可可花瘿病菌（*A. rigidiuscula*）菌株2268-2-2-3，同时对番荔枝果实上的病斑进行了描述。此外，本研究的致病性测定结果表明该菌株能够侵染番荔枝果实，形成黑褐色不规则病斑，本研究中菌株能否侵染番荔枝果树需进一步研究。除本次截获，2013年宁波口岸从日本进境罗汉松种苗上截获过可可花瘿病菌（*A. rigidiuscula*）[17]，2017年重庆口岸从越南进境番荔枝果实上也截获过该病菌。以上截获表明，可可花瘿病菌（*A. rigidiuscula*）分布较为广泛，容易通过水果、种苗等进行传播。

据统计，仅2020年我国进口水果数量高达630万吨。以往水果中疫情的截获多限于昆虫类，如地中海实蝇（*Ceratitis capitata*）、橘小实蝇（*Bactrocera dorsalis*）、南洋臀纹粉蚧（*Planococcus lilacius*）、大洋臀纹粉蚧（*Planococcus minor*）等[30-32]，水果上真菌疫情截获较少。由于水果是一种鲜活类产品，对货架期要求较高，在口岸通常要求较短检疫通关时间，而现行口岸检疫性真菌检测鉴定标准方法多要求进行分离培养，以此来进行结果判定，因此检测时间和周期都较长。以可可花瘿病菌检疫鉴定为例，带病植物材料、带菌土壤是病害传播的重要载体，准确、灵敏、快速的检测方法是严格执行口岸检疫措施及研究病害防控措施的有力工具。国内外现有关于可可花瘿病菌的研究，多集中于新病害报道、种类鉴定及防治方面[17,33]，而本研究表明该病菌EF1A基因片段具有较大变异性，有望利用该片段建立该病菌的快速检测方法。

近年来，随着对台贸易的不断扩大，大量台湾水果进入大陆，在满足国内市场需求同时也带来了一些生物安全隐患。如口岸检疫部门曾从来自台湾水果中截获过葡萄苦腐病菌（*Greeneria uvicola*）和牛眼果腐病菌（*Neofabraea kienholzii*）等检疫性真菌[34-35]。以上情况表明来自台湾地区水果具有一定检疫风险，需要加强口岸检疫工作，保护我国生物安全。

致谢：感谢广州海关技术中心王卫芳研究员在样品采集以及种类鉴定中给予的指导与帮助！

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