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福建砂梨急性花枯病病原菌的分离与鉴定

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摘 要：【目的】明确近年在福建砂梨产区新发生的梨急性花枯病的病原菌种类。**【方法】**从产区采集显现急性花枯症状的‘翠冠’梨病样品进行组织分离，并利用形态学和分子生物学相结合的方法对获得的菌株进行种类鉴定和致病性验证。**【结果】**通过组织分离和纯化，并根据其菌落形态特征从福建砂梨急性花枯病样品中获得31个刺盘孢属（*Colletotrichum*）菌株。选取其中的14个代表菌株进行形态学观察和多基因（ITS、*ACT*、*TUB*、*CHS-1*及*GAPDH*）系统发育分析的结果显示，它们均为松针刺盘孢（*C. fioriniae*）。在一周花龄的砂梨（品种‘丰水梨’）、西洋梨（品种‘三季梨’）和秋子梨（品种‘南果梨’）的离体花序上喷雾接种代表菌株（CFZH1、CFZH6、CFZH15）分生孢子悬浮液的结果显示，均产生有急性花枯症状，平均发病率分别95.8%、63.9%和81.3%。**【结论】**福建砂梨产区导致梨急性花枯病的病原菌为松针刺盘孢（*C. fioriniae*）。本研究是福建砂梨产区松针刺盘孢引起梨花枯死的首次报道。

关键词：砂梨；福建；急性花枯；松针刺盘孢

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Isolation and identification of the pathogen causing acute blossom blight of sand pear (*Pyrus pyrifolia*) in Fujian

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Abstract:【Objective】In recent years, a devastating fungal disease that damages flowers of sand pear (*Pyrus pyrifolia* Nakai), known locally as acute blossom blight of pear, has occurred in sudden outbreaks in Fujian province of China, causing severe wilting of petals and peduncle, rotting of young fruit, and resulting in economic loss. This study was carried out on identifying the species of pathogen causing acute blossom blight in *Pyrus pyrifolia* in Fujian province of China.【Methods】The occurrence and damage of the disease in the field was investigated, and damaged flower cluster samples from the diseased trees showing symptoms of acute blossom blight were collected. Diseased tissues (neighboring the asymptomatic regions) were cut into small pieces (4-5 mm^2) after surface sterilization (75% ethanol for 45 s, washed three times in sterile water and dried on sterilized filter paper). Four single colonies of each isolate were picked up with a sterilized needle (insect pin, 0.5 mm diam.) and transferred onto potato dextrose agar (PDA, 20% diced potato, 2% glucose, 1.5% agar, and distilled water) plates and incubated at 28 ℃ in the dark. Pure cultures were stored in 25% glycerol at －80 ℃. Representative isolates were chosen based on colony cultivation traits and morphological characteristics. Colony diameters were measured daily to calculate their mycelial growth rates ($mm \cdot d^{-1}$). The shape, color, and density of colonies were recorded after ten days. Moreover, the shape and color of conidia were observed under light microscopy (Olympus BX63, Japan) after 15 days. Multiple sequences of concatenated *ACT,*

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TUB2, CHS-1, GAPDH, and ITS sequences were aligned using MAFFT v.7 with default settings, and if necessary, manually adjusted in MEGA v.5.2.2. Maximum-likelihood (ML) was used to construct phylogenies using IQ-TREE. The 14 representative isolates of *Colletotrichum* sp. were selected for pathogenicity tests with a spore suspension on detached pear flowers (approx. 1-wk-old) of *P. ussuriensis* 'Nanguoli', *P. pyrifolia* 'Hohsui', and *P. communis* 'Guyot'. Briefly, the conidiospore suspension with a concentration of 1×10^6 conidia per mL was sprayed on the surface of flower clusters without being unwounded, and sterile water was used in parallel as a control. Each treatment was performed with three independent repeats. After inoculation, the preservative film was wrapped to keep moisture and cultured in the greenhouse at 25 ℃ with a 12/12 h light/dark photoperiod. The humidity was maintained at 85%. Then the symptoms were observed continuously.【Results】The petals showed pale yellow wet lesions at first, and then expanded to the whole petals. The color of the lesions turned dark yellow, and then they became black-brown rotted. The peduncles were dark brown and large sunken rot lesions on fruits in the process of time. The occurrence of the disease was unlimited, and the lesions were large and irregular, which can extend to the whole leaf, causing the whole leaf to fall black and brown. Pear acute blossom blight could lead to substantial economic losses due to excessive fruit rot, or the severe wilting of blossom. A total of 31 *Colletotrichum* strains were isolated from the damaged flower cluster samples according to colony morphological characteristics. Colonies were light red in the center and pale white on the margin cultured for ten days. Conidia with mass orange color produced on the PDA plate after 15 days. Conidia were hyaline, smooth-walled, aseptate with both ends slightly acute under the light microscopy. But no sexual organ was found. The 14 representative strains together with 17 reference strains from previously described species were subjected to multi-locus phylogenetic analyses with concatenated *ACT, TUB2, CHS-1, GAPDH,* and ITS sequences. The results showed that the strains isolated from the damaged flower cluster samples belonged to *C. fioriniae*. The conidiospore suspensions of representative strains (CFZH1, CFZH6, CFZH15) were sprayed on the surface of the clusters of three species of pears (*P. ussuriensis*, *P. pyrifolia* and *P. communis*), which could cause the symptoms of acute blossom blight within 3 days. And the average rates of incidence were 81.3%, 95.8%, and 63.9%, respectively, of which *P. pyrifolia* had the highest incidence rate compared with other two species of pears (*P. ussuriensis* and *P. communis*), and no lesions developed on the flower clusters as control. Moreover, the symptoms caused by inoculation in the three species of pears were consistent with those of *P. pyrifolia* in the field. These results showed that the acute blossom blight in *P. pyrifolia* in Fujian province was caused by *C. fioriniae*.【Conclusion】In this study, we employed morphological and multi-locus phylogenetic analyses, along with pathogenicity tests according to Koch's postulates to identify the species of *Colletotrichum* causing acute blossom blight of sand pear. It is the first report that *C. fioriniae* is the pathogen of pear acute blossom blight, which may supply new useful information for understanding the pathogenic diversity of *Colletotrichum*. This study provides a theoretical basis for the effective control of the acute blossom blight in Fujian province, China.

Key words: *Pyrus pyrifolia*; Fujian province; Acute blossom blight; *Colletotrichum fioriniae*

近些年来，随着我国梨生产规模的扩大，产区病害的发生危害日趋严重，病原菌种类不断增多，已成为影响其产业健康发展的主要因子之一[1]。由于我国梨产区的地理跨度大，各地的气候条件差异明显，且各地区栽培的梨品种不同，因此导致了病害的多样性。福建为我国南方重要砂梨产区，现有面积2.27万 hm^2。近两年福建建宁等地梨树花期发现大量的花瓣变褐、萎蔫，迅速腐烂枯死现象，常因此对部分梨园产量构成严重影响。

梨树病害种类较多，据报道有黑斑病、黑星病、

轮纹病、炭疽病、腐烂病、锈病、白粉病、疫腐病、黑胫病等30余种，而且同种病原菌可引起不同症状的病害，如恶疫霉（*Phytophthora cactorum*）可导致梨果实腐烂和梨树干基部腐烂[2]。某些同属不同种病原菌又可引起相同症状的病害，如葡萄座腔菌 *Botryosphaeria dothidea*、*B. rhodina*、*B. parva* 和 *B. obtusa* 均可导致梨干腐病[3]。对于梨树花枯国内外已有研究报道，由 *Erwinia amylovora* 引起的梨火疫病[4]和由 *Pseudomonas syringae* pv. *syringae* 引起的梨花枯病[5]均可导致梨花枯死。前者在我国至今尚未发生，被列入植物检疫名单，后者仅在浙江省杭州市郊区发现，但这两种病害均为细菌性病害。而近年来福建等地所发生的急性花枯病害从田间病状表现不具有细菌性病害特征，疑为其他病原所致。因此，准确鉴定福建砂梨花期发生病害的病原菌对于了解该病害流行规律及有效控制病害的发展非常重要。

笔者于2017—2018年对福建建宁该病果园发生危害状况进行调查，采集病样进行病原菌分离，采用形态学及分子生物学相结合的方法对病原菌进行鉴定并利用柯赫氏法则（Koch's postulates）验证，以明确引起砂梨急性花枯病的病原菌种类，为该病的有效防控提供可靠的理论依据。

1　材料和方法

1.1　材料

2019年3月下旬，在福建省建宁县从急性花枯病发生最严重的溪口镇梨园采集'翠冠'梨病样品，按东西南北中五个方位各选取3株花序受害的梨树，每株树上各取2个带有受害花序的枝条，用塑料袋分别包装。考虑到田间显症明显的花序很快枯死，易污染腐生菌，带回实验室后再从30个枝条上各挑选1~2个初显症状的花序，共选取40个花序用作病菌分离样品。

1.2　方法

1.2.1　*病原菌的分离*　切取病样花序梗病健交界处的4~5 mm^2表皮组织块放入75%乙醇消毒45 s，然后在无菌水中漂洗2次，取出植物组织放在灭菌的滤纸上晾干后，置于PDA培养基上，在28 ℃恒温培养箱中黑暗培养2 d后，挑取小块未污染的菌丝块移至新的PDA培养基上培养5 d[6]。挑取两块菌丝块分别置于PDA斜面4 ℃保存和25%甘油中−80 ℃保存备用。

1.2.2　*病原菌形态特征观察*　挑取培养5 d菌落边缘的菌丝块（直径5 mm），转接至无菌的PDA培养基中央，置于28 ℃、黑暗条件下的智能光照培养箱中培养。每个菌株重复3皿。每天记录菌落在培养基上的生长情况（菌落的颜色、高度、边缘形态、菌丝的疏密程度及产孢情况）并测量菌落直径。此外，在体视显微镜（SZX16，Japan）下观察分生孢子堆特征及光学显微镜（Olympus BX63，Japan）下观察分生孢子形态。

1.2.3　*分子鉴定*　将供试菌株的菌丝块放置在铺有灭菌玻璃纸的PDA培养基中央，置于28 ℃、黑暗条件下的智能光照培养箱中培养，6 d后收集菌丝。采用CTAB法提取每个分离物的基因组DNA[7]，并通过1.2%琼脂糖凝胶电泳后检测DNA质量。使用引物对ACT-512F/ACT-783R、T1/Bt2b、CHS-79F/CHS-345R、GDF1/GDR1和ITS4/ITS5分别对供试菌株的 *ACT*、*TUB2* 和 *CHS-1* 基因的部分序列、*GAPDH* 200 bp的内含子以及ITS序列进行PCR扩增。具体引物信息见表1[8-10]。PCR反应体

表1　用于扩增 *ACT*、*GAPDH*、ITS、*TUB2* 和 *CHS-1* 基因序列的引物

Table 1　List of primers used for *ACT*，*GAPDH*，ITS，*TUB2* and *CHS-1* sequences amplification

扩增区域 Target	引物名称 Primer	引物序列 Sequence (5' to 3')	退火温度 Annealing temperature/℃
ACT	ACT-512F	ATG TGC AAG GCC GGT TTC GC	59
	ACT-783R	TAC GAG TCC TTC TGG CCC AT	
GAPDH	GDF1	GCC GTC AAC GAC CCC TTC ATT GA	59
	GDR1	GGG TGG AGT CGT ACT TGA GCA TGT	
ITS	ITS-4	TCC TCC GCT TAT TGA TAT GC	56
	ITS-5	GGA AGT AAA AGT CGT AAC AAG G	
TUB2	T1	AAC ATG CGT GAG ATT GTA AGT	58
	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	
CHS-1	CHS-79F	TGG GGC AAG GAT GCT TGG AAG AAG	58
	CHS-345R	TGG AAG AAC CAT CTG TGA GAG TTG	

系和扩增程序参考前人的方法[11-12]。使用PCR产物纯化试剂盒(生工生物工程(上海)股份有限公司)纯化PCR扩增得到的目的片段,并将纯化产物送至该公司进行测序。

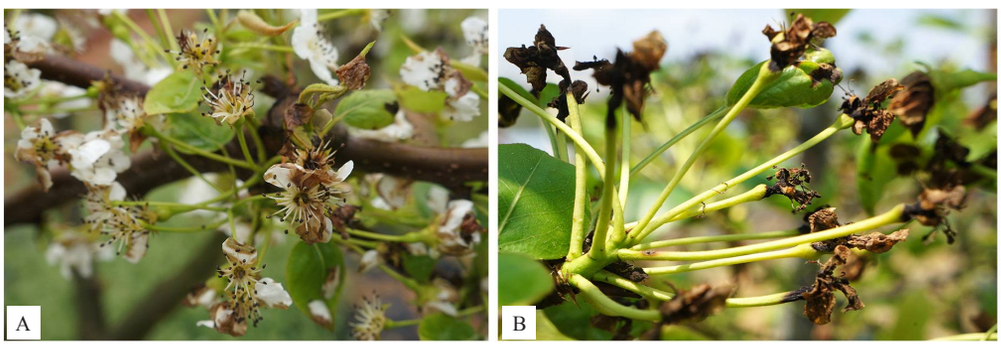
1.2.4 系统进化分析 采用DNAMAN 9.0软件对每个测序菌株的扩增核苷酸序列进行多重比对,再用默认设置的MAFFT v.7[13]对序列进行多重比对,必要时用MEGA7[14]对序列进行手工校正。将比对好的基因序列按照*ACT*、*TUB2*、*CHS-1*、*GAPDH*和ITS的顺序首尾相连进行拼接[15]。基因序列合并的数据集共有1 605个碱基(包含比对产生的空缺),使用IQ-TREE对合并的数据集进行遗传进化分析,采用最大似然法(Maximum-likelihood)并选用GTR+G核苷酸替代模型构建系统进化树,重复1 000次。

1.2.5 致病性测定 以1周花龄的砂梨(品种'丰水梨')、西洋梨(品种'三季梨')和秋子梨(品种'南果梨')为材料,进行离体无伤喷雾接种[11]。即应用手提加压小喷壶,将代表菌株CFZH1、CFZH6、CFZH15的分生孢子悬浮液(浓度为10^6个•mL^{-1})直接喷于离体花序表面进行无创伤接种,并以无菌水接种为对照。每个装有水的锥形瓶中插入3枚花朵离体梨花,并在外层裹上一层保鲜膜进行保湿,每个处理设置3次重复,每个品种接种48~72枚花朵。接种期间温室内培养条件为25 ℃、12/12 h光暗交替,并维持接种小环境的湿度为85%左右,期间观察发病情况,并观察发病症状是否与田间症状相同。

2 结果与分析

2.1 梨急性花枯病的田间症状表现

田间调查结果显示,梨急性花枯病在'翠冠''晚翠''翠玉''新玉''黄花'等砂梨品种上均可发生,主要危害花瓣、花梗,也危害幼果(图1)。最初花瓣边缘呈浅黄色水渍状,随后扩展全瓣,病斑颜色转为黄色、深黄色,后呈黑褐色腐烂状萎蔫,覆盖幼果并继续在幼果上蔓延,直到全果、果柄呈黑褐色,病斑呈大块黑色凹陷。受害的花瓣、幼果脱落掉于幼叶上后诱发叶片发病,发生部位不限,病斑大而无规则,可扩展到至全叶,导致全叶呈黑褐色脱落。该病发生严重时,花朵受害可达95%,导致绝产绝收、树势衰弱。



A. 发病初期;B. 发病后期。

A. Symptom in the early stage;B. Symptom in the later stage.

图1 砂梨急性花枯病的田间症状表现

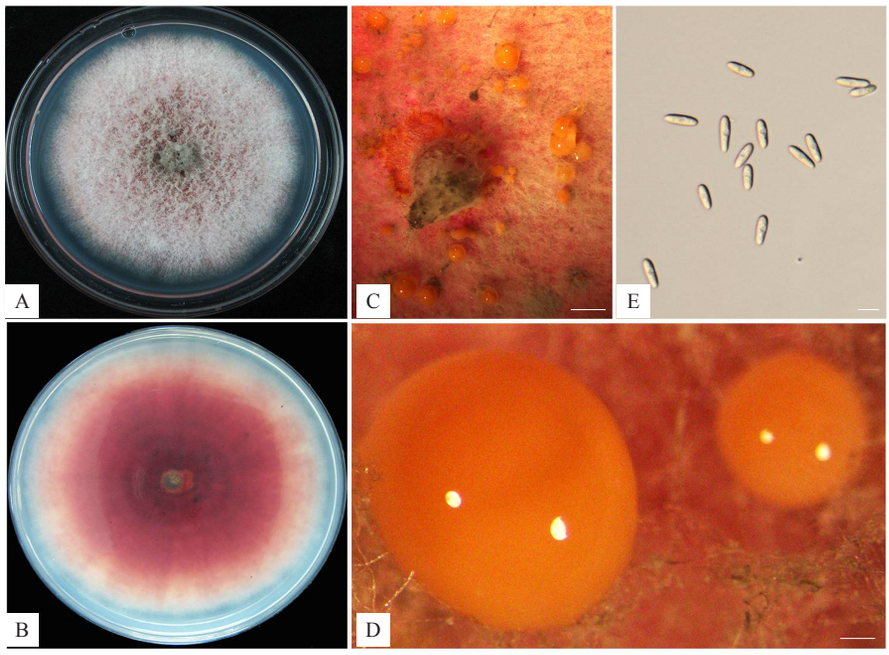
Fig. 1 The field symptoms caused by acute blossom blight on *Pyrus pyrifolia*

2.2 病原菌培养特性与形态学特征

本研究对从福建省建宁县溪口镇梨园采集的40份梨品种为'翠冠'的急性花枯病样品中分离获得的纯化菌,根据其菌落形态特征共选出31个刺盘孢(*Colletotrichum*)菌株。将这些菌株放置在28 ℃、黑暗条件下的PDA培养基中培养10 d后,可观察到菌落正面中央浅红色,背面深红色,颜色从中央向边缘递减(图2-A、B);随着时间的延长,棉絮状的气生菌丝逐渐变灰。培养15 d后肉眼可见橘黄色分生孢子堆,在体视显微镜下观察到其着生在PDA培养基上,表面光滑(图2-C、D)。吸取少量分生孢子悬浮液置于载玻片上,在光学显微镜可看到分生孢子呈梭状,透明,光滑,无隔膜,两端渐尖(图2-E)。培养期间未见有性态;根据其培养特性和形态学特征,可初步判断分离获得的菌株为刺盘孢属菌(*Colletotrichum* sp.)。

2.3 多基因系统发育分析

对从病花序样品中分离获得的31个刺盘孢



A、B. 分别为 PDA 平板上培养 10 d 的正面和背面菌落形态；C、D. 分生孢子堆；E. 分生孢子。A、B、C、D. 取自菌株 CFZH11；E. 取自菌株 CFZH3；—标尺：C、D = 5 mm，E = 20 μm。

A, B. Front and back views of 10-d-old PDA culture; C, D. Conidiomata; E. Conidia; A, B, C, D. Isolate CFZH11; E. Isolate CFZH3; — Scale bars: C,D= 5 mm，E = 20 μm.

图 2　病原菌的形态特征

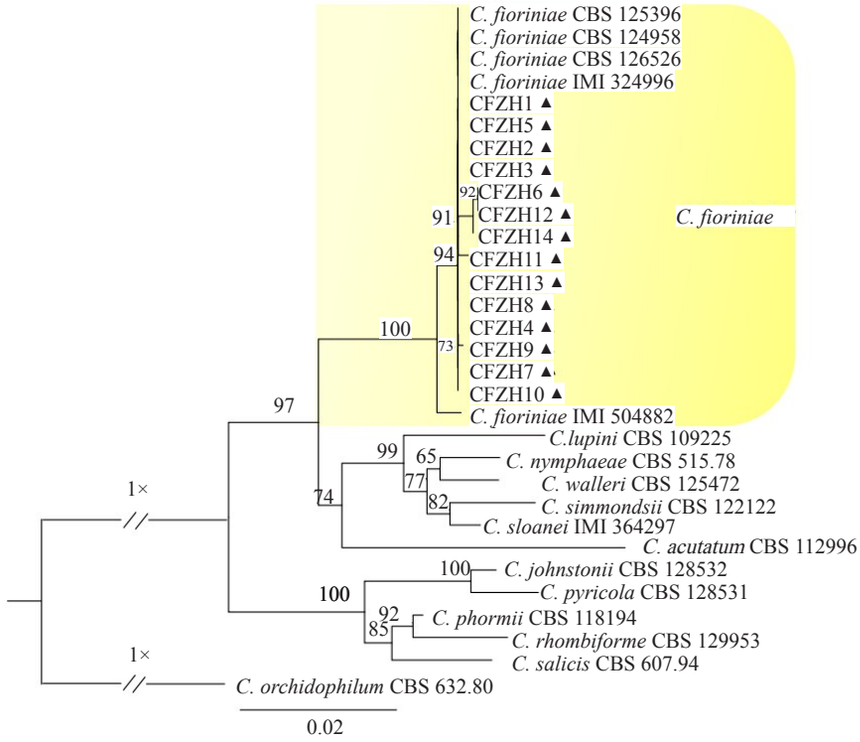
Fig. 2　Morphological characteristics of pathogens

（*Colletotrichum*）菌株中的14个菌株，提取基因组DNA，进行ITS、*ACT*、*GAPDH*、*TUB2*和*CHS-1*序列的PCR扩增，得到大小为579~583 bp、238~247 bp、250~256 bp、740~760 bp和275~291 bp的特异性片段。根据在福建建宁从砂梨品种‘翠冠’上采集的14个菌株各5个基因的测序结果（表2），在GenBank（http://www.ncbi.nlm.nih.gov）数据库中进行Blast比对后，选取17个刺盘孢属真菌的相应基因区域序列作为参考序列，其中以*C. orchidophilum* CBS 632.80作为外群，进行多基因系统发育分析。结果显示，本研究中的14个菌株与*C. fioriniae*聚在同一分支（图3）。由此，可进一步证实本研究所分离获得的菌株

表 2　本研究用于系统发育分析菌株的采集信息及 GenBank 登录号

Table 2　Collection details and GenBank accession numbers of isolates used phylogenetic analysis in this study

采集地点 Collection location	分离品种 Isolated variety	菌株 Strain	基因序列登录号 GenBank accession numbers				
			ITS	ACT	GAPDH	TUB2	CHS-1
福建建宁 Jianning, Fujian	砂梨（品种翠冠） *P. pyrifolia* Cuiguan	CFZH1	MT607638	MT625872	MT625900	MT625886	MT625914
		CFZH2	MT607639	MT625873	MT625901	MT625887	MT625915
		CFZH3	MT607640	MT625874	MT625902	MT625888	MT625916
		CFZH4	MT607641	MT625875	MT625903	MT625889	MT625917
		CFZH5	MT607642	MT625876	MT625904	MT625890	MT625918
		CFZH6	MT607643	MT625877	MT625905	MT625891	MT625919
		CFZH7	MT607644	MT625878	MT625906	MT625892	MT625920
		CFZH8	MT607645	MT625879	MT625907	MT625893	MT625921
		CFZH9	MT607646	MT625880	MT625908	MT625894	MT625922
		CFZH10	MT607647	MT625881	MT625909	MT625895	MT625923
		CFZH11	MT607648	MT625882	MT625910	MT625896	MT625924
		CFZH12	MT607649	MT625883	MT625911	MT625897	MT625925
		CFZH13	MT607650	MT625884	MT625912	MT625898	MT625926
		CFZH14	MT607651	MT625885	MT625913	MT625899	MT625927



三角形表示为本研究获得的代表菌株。

Triangles indicate representative strains obtained by this study.

图3 本研究获得的代表菌株与参考菌株的多基因(*ACT*、*TUB2*、*CHS-1*、*GAPDH* 和 ITS)序列采用最大似然法构建的系统进化树

Fig. 3 Maximum-likelihood inference phylogenetic tree obtained from the combined *ACT*, *TUB2*, *CHS-1*, *GAPDH* and ITS sequences alignments of representative strains obtained by this study and reference strains

均为松针刺盘孢(*C. fioriniae*)。

2.4 致病性验证

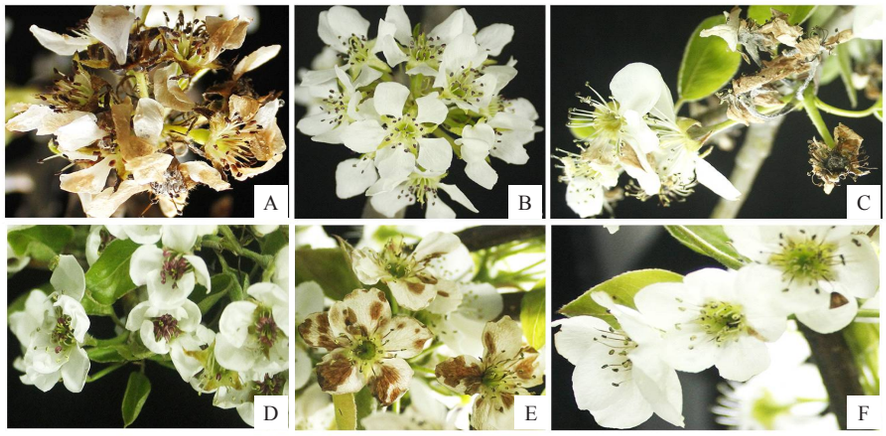
在1周花龄的砂梨(品种'丰水梨')、西洋梨(品种'三季梨')和秋子梨(品种'南果梨')的离体花序上喷洒接种代表菌株(CFZH1、CFZH6、CFZH15)分生孢子悬浮液1 d后均开始发病,2~3 d,梨花枯病菌在花上形成水渍状、黄色病斑,后期花瓣腐烂萎蔫,病斑逐步蔓延至花梗,5~7 d后,整株呈枯死状;对照均未发病。3个梨种的花发病症状均与砂梨田间症状一致(图4)。于接种后第3天调查发病率。结果(表3)显示,砂梨、西洋梨和秋子梨的平均发病率分别为95.8%、63.9%和81.3%,可看出砂梨相较于其他品种发病率最高。

对以上接种发病的组织按品种随机取样进行病原菌再分离,结果显示再分离出的病菌与接种菌株一致。

3 讨 论

本研究表明,发生在福建建宁等地的砂梨急性花枯是由松针刺盘孢(*C. fioriniae*)所致。松针刺盘孢在分类上归属于尖孢刺盘孢(*C. acutatum*)复合种[12],该复合种包含多个种,其多基因(*ACT*、*TUB2*、*CHS-1*、*GAPDH*和ITS)系统发育与形态学特征存在差异[16],可为危害梨、苹果、桃、杏、鳄梨、猕猴桃、杧果、蓝莓等多种植物的叶片和果实[17-24],2019年在巴西又发现一种新的尖孢刺盘孢(*C. nymphaeae*)可以导致梨花瓣和花梗枯死及果实腐烂[25],本研究是福建砂梨产区松针刺盘孢(*C. fioriniae*)导致梨花枯死的首次报道,研究结果为了解尖孢刺盘孢的致病多样性提供了新的信息。

根据报道,松针刺盘孢(*C. fioriniae*)的不同菌株可聚集成两个亚分支(subclade Ⅰ和subclade Ⅱ),二者的序列之间存在差异[17]。本研究从显现急性花枯症状的花序中所分离获得的松针刺盘孢菌株均聚集在subclade Ⅱ,而本实验室从显现灰色病斑症状的梨叶中分离获得的松针刺盘孢菌株,则聚集在subclade Ⅰ和subclade Ⅱ[12]。从本研究可看出,由松针刺盘孢(*C. fioriniae*)引起的砂梨急性花枯,其症



A、B. 秋子梨（品种：南果梨）；C、D. 砂梨（品种：丰水梨）；E、F. 西洋梨（品种：三季梨）；症状拍摄的时间为接种后第 3 天；A、C、E. 松针刺盘孢分生孢子悬浮液喷洒梨花的症状图；B、D、F. 无菌水喷洒梨花的症状图。

A，B. *P. ussuriensis* cv. Nanguoli; C，D. *P. pyrifolia* cv. Hohsui; E，F. *P. communis* cv. Guyot; The symptoms caused by these species were photographed at 3 dpi; A，C，E. The symptoms of spore suspensions of *C. fioriniae* spray pear flowers; B，D，F. The symptoms of sterile water spray pear flowers.

图 4　对 3 种梨的离体花序喷雾接种松针刺盘孢代表菌株分生孢子悬浮液后第 3 天产生的症状

Fig. 4　Symptoms of inflorescences on three species of pears after three days of spray inoculation with *conidia* suspensions of representative strains of *C. fioriniae*

表 3　松针刺盘孢分生孢子悬浮液喷雾接种 3 种梨的离体花序后第 3 天的发病率

Table 3　Incidences of diseased inflorescences on three species of pears after three days of spray inoculation with conidia suspensions of representative strains of *C. fioriniae*

种类 Species	品种 Cultivar	发病次数/接种花朵数 Number of diseased /inoculated flowers			平均发病率 Average incidence rate/%
		I	II	III	
砂梨 *Pyrus pyrifolia*	丰水梨 Hohsui	24/24	22/24	23/24	95.8
西洋梨 *Pyrus communis*	三季梨 Guyot	15/24	18/24	13/24	63.9
秋子梨 *Pyrus ussuriensis*	南果梨 Nanguoli	13/16	15/16	11/16	81.3

状表现与由梨火疫病菌（*E. amylovora*）和丁香假单胞杆菌丁香致病型（*P. syringae* pv. *syringae*）两种细菌引起的梨花枯死存在明显的差异，后两种在湿度较高时病斑处会溢出菌脓，镜检均可见喷菌现象，而松针刺盘孢（*C. fioriniae*）引起的砂梨急性花枯在田间没有这种现象，但其病情变化又与这两种细菌病害十分相似，在气温和湿度适宜情况下均可在较短的时间内造成梨花迅速枯死。因此，对松针刺盘孢的侵染菌源、途经、寄主范围及其致病性分化也有待深入研究。

本研究结果显示，在温室接种条件下，松针刺盘孢使砂梨、秋子梨和西洋梨均可产生急性花枯症状，这就暗示在我国秋子梨和西洋梨产区也存在与砂梨产区相同的由松针刺盘孢引起急性花枯的可能，因此，进一步研究明确松针刺盘孢在福建省不同梨产区的地理分布及致病力分化状况，可为梨产区有效防治急性花枯病提供科学依据。

4　结　论

福建砂梨产区导致梨急性花枯病的病原菌为松针刺盘孢（*Colletotrichum fioriniae*），本研究是福建砂梨产区松针刺盘孢引起梨花枯死的首次报道。

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