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来源于产黄青霉病毒科成员的分离物对梨轮纹病菌生长及其致病力的影响

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摘 要:【目的】明确产黄青霉病毒科成员(*Botryosphaeria dothidea* Chrysovirus 1, BdCV1)对寄主梨轮纹病菌(*Botryosphaeria dothidea*)菌株生物学性状的影响,证实单独感染BdCV1梨轮纹病菌分离株是弱毒菌株;以期确定BdCV1是否为引起梨轮纹病菌弱致病力的单一因子。【方法】从前期弱致病力复合感染双分体病毒科成员BdPV1和BdCV1的梨轮纹病菌LW-1中经多代菌丝分离获得的疑似仅单独感染BdCV1分离株(命名为LW-C)为材料;采用dsRNA检测、RT-PCR,鉴定LW-C菌株中仅携带BdCV1。采用菌丝块接种于PDA培养基以及3针针刺法接种梨果实,观察其菌落形态以及发病情况,测定菌落生长速度和致病性,明确LW-C生物学特性。将LW-C对无毒菌株Mock进行水平转染,检测其传染后衍生菌株的dsRNA并测定其生物学特性。【结果】证实了LW-C仅感染BdCV1,具有弱毒特性;水平转染结果明确了BdCV1因子对梨轮纹病菌的菌落形态、生长速度有抑制作用,对寄主有弱致病力。【结论】确认了BdCV1是引起梨轮纹病菌致病力衰退的主要因子,为真菌病毒防治果树轮纹病害提供了新颖的生防材料。

关键词:梨轮纹病害;葡萄座腔菌;产黄青霉病毒科;双分体病毒科;真菌病毒;双链RNA;致病力;生物防治

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Effect of *Botryosphaeria dothidea Chrysovirus 1* isolate belonging to the Chrysoviridae family on growth and pathogenicity of the *B. dothidea* strain infection in pears

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Abstract:【Objective】To identify LW-C infection by only *Botryosphaeria dothidea Chrysovirus 1* (designated as BdCV1), an attenuated strain, and evaluate the effect of BdCV1 on the biological features of the phytopathogenic fungus of *B. dothidea*. This study aims to answer if BdCV1 is responsible for the attenuated hypovirulence of the phytopathogenic fungus *B. dothidea*.【Methods】In this study, the suspected strain (designated as LW-C) only infected with BdCV1 was obtained as the test material, from the hypovirulence isolate of LW-1 coinfected with BdCV1, a member of the family of Chrysoviridae, and *B. dothidea Partitivirus 1* (designated as BdPV1), and a member of the Partitiviridae by the hyphal tipping technique. In order to confirm LW-C only infection with BdCV1, isolated from the LW-1 strain, dsRNA detection, RT-PCR and sequence analysis were used. To assess the biological features of LW-C strain, mycelial agar plugs from the colony margin of a freshly cultured LW-C strain, LW-1, LW-P and HL-1 as controls, respectively, were placed on a PDA in petri dishes (9 cm in diameter) and incubated at 25 °C in the darkness for determination of the mycelial growth rate and for phenotype observation. The virulence was detected by inoculating detached fruits of *Pyrus pyrifolia* 'Huangguan'. The lesions developed from the strains

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were measured and recorded at 7 days post-inoculation (dpi) for the inoculated fruits. The horizontal transmission of BdCV1 infecting the LW-C strain was assessed by LW-C and mock dually cultured. The derivates from Mock were biologically characterized and their virulence was determined as described. 【Results】 BdCV1 only infection with LW-C was confirmed by the detection of dsRNA patterns, RT-PCR for *RdRp* genes and sequencing analysis from dsRNA2 of BdCV1. The biological characteristics of the LW-C strain were analyzed in comparisons with *B. dothidea* strong virulent strains of LW-P and HL-1, and the attenuated strain LW-1, respectively. The results showed that LW-C exhibited an abnormal phenotype with irregular and sectored colony margins. The growth rate of strain LW-C cultured on the PDA (25 ℃ in darkness) was slower with 4.29 mm·d^{-1}, almost equal to that of LW-1, whereas the growth rates for LW-P and HL-1 were 22.05 and 15.5 mm·d^{-1}, respectively. In addition, it was difficult to produce spores for the LW-C. More importantly, the LW-C strain exhibited no or very weak virulence on 'Huangguan' fruits, equal to that of LW-1 with a lesion size of less than 5.0 mm, respectively, whereas the lesion sizes were 38.65 mm and 34.56 mm on fruits for LW-P and HL-1 of the *B. dothdiea* strains. The hypovirulent characterization of the LW-C and the horizontal transmission of dsRNA with LW-C to mock (25 ℃ in darkness) were determined. In contact cultures between strains LW-C and Mock, the Mock grew rapidly and covered the entire plates after 3 days, while the LW-C strain grew much slower. Five mycelial derivative subisolates selected randomly (designated as M-13, M-16, M-17, M-21 and M-22) were obtained from five derivates of the Mock in five contact cultures of LW-C/mock at 3 dpi. Isolates of M-13, M-16, M-17 and M-22 were similar to the LW-C strain as the donor in the mycelial growth on the PDA (1.1 to 3.9 mm·d^{-1}), morphological features of abnormal phenotype with sectored regions, and weak pathogenicity on the pears (fruits of 'Kuerle Xiangli' and 'Huangguan' had lesions of 1.3 to 5.1 mm and 3.1 to 5.9 mm in diameter, respectively), which was apparently different from that of Mock in the growth rate of 18.5 mm·d^{-1}, and the lesions of 29.7 and 32.3 mm on fruits of 'Kuerle Xiangli' and 'Huangguan', respectively. The presence of dsRNAs patterns in derivates of M-13, M-16, M-17 and M-22 were detected by 1.2% agarose gel electrophoresis, which were in accordance with that of the BdCV1 from LW-C. Therefore, BdCV1 has inhibitory effects on LW-C, which can be horizontally transmitted to mock through a hyphal contact culture. However, the other subisolate of M-21 in comparison with the parental strain Mock showed no significant difference in the growth rate of the 13.6 mm·d^{-1} or virulence on the pear fruits of 'Kuerle Xiangli' at 5 dpi and 'Huangguan' at 8 dpi with lesions of 23.6 and 31.6 mm, respectively. Lots of conidia were observed on the PDA palate at 7 d for the M-21 strain. Examination by agarose gel electrophoresis disclosed no dsRNA existence in the M-21, revealing that BdCV1 from LW-C could not transmit to Mock. 【Conclusion】 It was demonstrated that LW-C isolated from the LW-1 strain infected only by BdCV1 resulted in significant alteration in the growth rate, virulence and phenotype of the phytopathogenic fungus *B. dothidea*. The obtained results support the suggestions that BdCV1 is responsible for the attenuated hypovirulence of the phytopathogenic fungus. Till now, this is the first report to confirm that BdCV1, belonging to a new member of the Chrysoviridae family, induced the hypovirulence of the phytopathogenic fungus *B. dothidea*. Therefore, the BdCV1 of *Chrysovirus* is a good candidate for the biological control of apple and pear ring spot diseases. In the future, it will be necessary to theoretically evaluate the transfection effect of BdCV1 infecting LW-C on the other *B. dothidea* strains and identify the molecular mechanism of the host hypovirulence associated mycovirus BdCV1. It will aim to provide new ideas and strategies for safe biological control of ring rot disease induced by *B. dothidea* strains in practice.

Key words: *Pear ring rot* disease; *Botryosphaeria dothidea*; Chrysoviridae; Partitiviridae; Mycovirus; dsRNA; Pathogenicity; Biological control

梨轮纹病由葡萄座腔菌[(*Botryosphaeria dothidea* (Moug:Fr) Ces & De Not)]引起,该病害也称粗皮病、瘤皮病或水烂病。梨轮纹病病原菌寄主范围广,除感染梨外,还能危害苹果、桃、李、杏、海棠、板栗、枣等多种果树;在我国梨产区普遍发生,造成大量烂果,枝干粗皮、溃疡及枝条枯死,导致树枝早衰,严重影响产量和品质,给果农带来巨大的经济损失[1-5]。

目前,对于果树轮纹病害的防控主要依靠化学防治,长期大量使用化学杀菌剂使果园生态环境恶化,存在食品安全隐患,同时果树容易产生抗药性。果实套袋技术对轮纹病起到了一定的防治效果,但对枝干危害的加重却因为其隐蔽性而更易造成不可挽回的危害和大范围流行,这些措施都未能有效控制病害的发生和流行[6-9]。轮纹病的危害近年来逐年加重,寻找新的、安全有效的措施来防治梨树轮纹病害显得尤为迫切和重要,未来开展梨轮纹病的生物防治方面的研究十分重要。弱毒相关病毒导致植物病原真菌群体的致病力衰退,可作为真菌病害生物防治的另一条重要途径[10-12]。多数真菌病毒对寄主没有显著性影响,不表现感染特征,如双分体病毒科(Partitiviridae)的大多真菌病毒对寄主生物特性及其致病力无任何影响,但少数真菌病毒对植物病原真菌寄主的表型有显著的抑制作用,可引起寄主致病力出现衰退,具有良好的生物防治潜能[13-14]。最早在板栗树上发现的弱毒菌株可以有效防治板栗疫病,以及DNA病毒防治油菜菌核病[10-12,15]。近年来在也有少量关于真菌病毒对果树上感染的真菌具有一定的生防效果的报道,如dsRNA病毒、白纹羽病菌病毒RnMRV1(*Rosellinia necatrix megabirnavirus 1*)和苹果腐烂病菌低毒力病毒VcHV1(*Valsa ceratosperma hypovirus 1*)分别具有控制果树白纹羽病害和苹果腐烂病害的潜力[11,16]。

笔者课题组前期首次分离获得1株菌落形态异常的梨轮纹病菌菌株,编号为LW-1分离株,该菌株生长速度慢、菌落扇变、色素形成异常、侵染寄主表现弱致病力,具有低毒特性[14]。经dsRNA检测发现该弱毒菌株LW-1复合感染2种dsRNA病毒,克隆获得其全长序列,鉴定一种为双分体病毒科新成员,命名为BdPV1(*Botryosphaeria dothidea Partitivirus 1*)。另一种病毒鉴定为产黄青霉病毒科新成员,命名为BdCV1(*Botryosphaeria dothidea Chrysovirus 1*),基因组内含4条dsRNA;水平传染及原生质体转染试验结果表明,BdPV1对衰退菌株LW-1生长速度变慢、弱致病力等生物学特性无任何相关性。前期研究尚未获得单独感染BdCV1分离株,笔者不清楚LW-1衰退现象的出现是BdPV1与BdCV1之间互作还是BdCV1单独作用导致,有待进一步研究。因此,本研究经单菌丝分离获得仅含BdCV1病毒的分离株,旨在分析其生物学特性,明确其对寄主梨轮纹病菌菌株生物学性状的影响,以期确定BdCV1是否为引起梨轮纹菌株弱致病力的单一因子,明确产黄青霉病毒可引起梨轮纹菌弱致病力,证实LW-C为弱毒菌株,深入挖掘BdCV1病毒的生物防治潜力,进一步评价其不同遗传背景下病毒的传染能力,为真菌引起果树轮纹病害的生物防治提供重要的生防资源。

1 材料和方法

1.1 材料

梨轮纹病菌分离株HL-1和LW-1分别分离自湖北省武汉市果树茶叶研究所砂梨种质资源圃'华梨一号'和'金水一号'枝干的梨轮纹病菌分离物。HL-1为强致病力菌株,不携带病毒。LW-1为弱致病力菌株,携带葡萄座腔菌产黄青霉病毒1(*Botryosphaeria dothidea Chrysovirus 1*, BdCV1)和葡萄座腔菌双分体病毒(*Botryosphaeria dothidea Partitivirus 1*, BdPV1)。LW-P为单菌丝分离自LW-1分离株,携带BdPV1,具有强致病力特性。Mock,分离自LW-1,不携带病毒,作为阴性对照。本研究中LW-C为LW-1菌株经多代分离培养,在其后代中疑似仅携带BdCV1的分离株,命名为LW-C分离株,需进一步鉴定并确认。

1.2 梨轮纹病菌菌丝中双链RNA(double-strand RNA, dsRNA)的提取

参照杨帆等[17]的真菌病毒dsRNA柱式分离法,提取梨轮纹病菌分离株dsRNA(专利号ZL201310072994.3)。主要步骤如下:刮取铺于玻璃纸固体PDA上培养的梨轮纹病菌株(每个菌株打取3~4个菌丝块接种于直径为9 cm皿中进行培养;LW-1和LW-C菌株均培养6 d,HL-1、LW-P和Mock菌株分别培养3 d,菌丝每皿0.3~0.5 g,液氮研磨,加入700 μL Buffer I,65 ℃水浴10~15 min,12 000 r·min^{-1}离心5 min;吸取上清,加入1.5倍体积的Buffer II,离心同上;于上清液中加入0.5倍体积的

无水乙醇,颠倒混匀,随之将其加入到吸附柱中,离心1 min;依次分别加入700 μL的Washing Buffer I和Washing Buffer II至吸附柱中洗脱,离心1 min;最后向吸附柱中央硅膜加入50 μL ddH_2O,回收获得的dsRNA,核酸酶处理后,电泳检测,观察其dsRNA特征条带,于-20或-80 ℃保存备用。

1.3　反转录聚合酶链式反应(reverse transcription-PCR, RT-PCR)

以2 μL dsRNA为模板,加入1 μL 6个碱基随机引物,7 μL ddH_2O并充分混匀,95 ℃水浴10 min后放入冰上3~5 min;加入4 μL 5×反转录缓冲液、0.5 μL RNA酶抑制剂(RRI)、0.5 μL反转录酶(M-MLV),加去离子ddH_2O水至20 μL,于37 ℃孵育1.5 h,最后于95 ℃ 3 min灭活反转录酶获得cDNA产物,放置于-20或-80 ℃备用。

参照GenBank上报道的真菌病毒BdCV1和BdPV1的*RdRp*和*cp*基因序列设计引物,具体引物序列信息见表1,用于病毒基因片段的PCR检测。扩增体系具体如下:2.5 μL 10×PCR buffer,1 μL dNTP,0.5 μL正向引物,0.5 μL反向引物,1 μL cDNA,0.25 U *Taq* DNA聚合酶,加ddH_2O(19.25 μL)至25 μL。

PCR扩增程序为:95 ℃预变性3 min,95 ℃变性30 s,55~58 ℃退火30 s,72 ℃延伸30~60 s,35个循环;72 ℃延伸10 min。

表1　扩增BdPV1和BdCV1的*RdRp*和*cp*基因的引物序列信息

Table 1　The information of primers used for amplification the genes of *RdRp* from BdPV1 and BdCV1, *RdRp* and *cp* from BdCV1, respectively

病毒基因名称 Gene name	引物序列(5'-3') Primer sequence (5'-3')	PCR产物片段大小 PCR product size/bp	引物位置 Location	GenBank登录号 GenBank accession number
BdPV1 RdRp	F:GGTGATGAGGACATGTCGAG	144	472~491	KF688740
	R:AAGGAAACCAACTGGACGAC		596~615	
BdCV1 RdRp	F:CGTCGTTCACCAACAGCATCC	186	2 174~2 194	KF688336
	R:ACTCGAACCCCATTACTTTACCG		2 359~2 337	
BdCV1 dsRNA2-1	F:CGCAAAAAAGAAGAAAAGGGG	867	1~21	KF688737
	R:TCACGTTCGACAACCCCG		850~867	
BdCV1 dsRNA2-2	F:ATCTGGATGTGGCCGCTG	658	790~807	KF688737
	R:AACAGCTCGTGGTTGCGT		1 447~1 430	
BdCV1 dsRNA2-3	F:CTCGGCCATTGTTGCCCT	538	1 032~1 321	KF688737
	R:TGCTTTGGTGCCCGCATA		1 841~1 824	
BdCV1 dsRNA2-4	F:CGACCCGACTTCACGCTG	979	1 795~1 821	KF688737
	R:ACACAATTTCTGGAGC		2 773~2 758	

1.4　梨轮纹病菌菌株生长速率测定

将供试梨轮纹菌菌株在PDA平板上活化4 d,用灭菌的打孔器(直径5 mm)在菌丝边缘打取菌丝块,转接到PDA平板中央,置于25 ℃、黑暗条件下培养。每个试验菌株重复3次以上,每隔24 h观察菌落形态,测量并记录菌落直径。

1.5　梨轮纹病菌菌株LW-C致病力测定

为明确LW-C菌株的致病性,以供试菌株LW-1、LW-P、HL-1以及空白PDA菌丝块为对照,采用离体果实进行接种。选取大小一致、健康、成熟的离体'黄冠'梨果,75%酒精消毒和无菌水擦洗干净后,在梨果实表皮上用经灭菌的接种针做力度一致的3针刺伤处理,将带有菌丝的一面平贴针刺点,以PDA培养基块为空白对照,每个菌株重复3次以上,用蘸有无菌水的灭菌棉花覆盖在菌丝块上保湿,放置于用保鲜膜封闭的白瓷盘中于恒温25 ℃进行保湿培养,每隔24 h观察发病情况,测量并记录梨果上产生病斑大小。

1.6　LW-C菌株的弱毒特性及dsRNA的水平传染试验

采用对峙培养的方法,测定LW-C菌株的弱毒特性及dsRNA的传染能力。将活化的LW-C菌株放置在PDA平板(距培养皿边缘1.5 cm)上培养3 d,在其菌丝块边缘接种Mock进行对峙培养(两菌丝块相距约1 cm),置于25 ℃培养,每隔24 h观察并记录培养菌株的菌落形态。培养3 d后,选取对峙培养所得的衍生菌株,检测其携带的dsRNA带型,分析其生长速度以及接种于离体'黄冠'和'库尔勒香梨'果实

进行致病力测定，分析LW-C携带dsRNA的水平传染效果，评价其弱毒特性。

1.7 数据分析

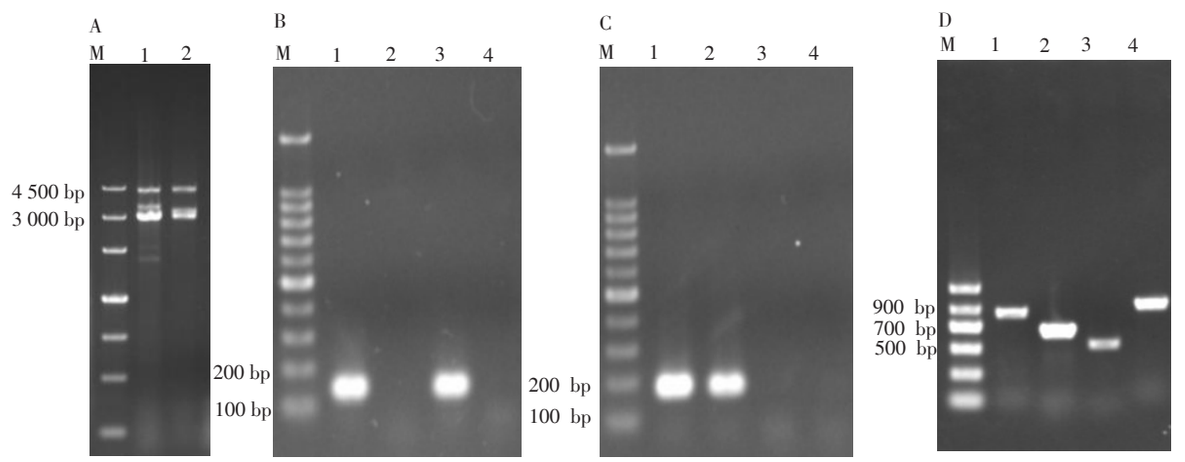
菌株生长速率、病斑直径的数据处理分别采用SPSS软件（21.0版本）中Duncan法做显著性检验，并采用Excel 2003作图。

2 结果与分析

2.1 LW-C菌株携带BdCV1的鉴定

2.1.1 LW-C携带BdCV1 4条dsRNA的测定 从LW-1菌株中多次进行分离纯化，获得了疑似携带BdCV1病毒的分离株，编号为LW-C分离株。采用dsRNA柱式分离法提取其菌丝dsRNA，经过核酸酶处理、1.2%琼脂糖凝胶电泳、EB染色后，LW-C菌株的dsRNA带型除不能清晰观察到BdCV1编码*cp*基因的dsRNA 2条带外，其余3条dsRNA条带与BdCV1对应的dsRNA带型一致（图1-A）。

2.1.2 LW-C携带BdCV1 *RdRp*和编码*cp*基因的dsRNA2全长扩增的PCR检测 为了进一步证实分离自LW-1菌株的LW-C分离株仅含BdCV1基因，分别设计BdCV1和BdPV1 *RdRp*和*cp*基因的特异检测引物（表1），对其进行RT-PCR检测。结果表明，LW-C和LW-1菌株PCR扩增获的BdCV1 *RdRp*特异性目标条带约200 bp（图1-C）；LW-P和LW-1菌株获得BdPV1 *RdRp*基因的特异扩增条带约150 bp（图1-B），进一步证实了LW-C菌株仅含有BdCV1；在电泳观察中，LW-1携带BdCV1的dsRNA2有时也观察不到目标条带，为了进一步证实LW-C菌株中是否存在dsRNA2，参照报道的BdCV1编码*cp*基因的dsRNA2全长序列设计4对特异检测引物（表1）对LW-C BdCV1全长dsRNA2进行RT-PCR检测，获得含有dsRNA2全长扩增的预期目标片段，分别为867、658、538和979 bp（图1-D），对其进行克隆以及序列测定，经拼接后全长序列同源性与LW-1中BdCV1 dsRNA2相似性高于97%。综合以上LW-C分离株的dsRNA鉴定、*RdRp*基因的RT-PCR检测及其dsRNA2序列测定结果，证实LW-C分离株仅携带BdCV1 4条dsRNA特征条带。



A. 梨轮纹菌株dsRNA 1.2%的琼脂糖凝胶电泳分析；M为DNA Maker III（天根公司），1和2分别来源于LW-1和LW-C菌株样品。B和C分别为BdPV1和BdCV1的*RdRp*基因的PCR电泳图；M为DNA 100 bp ladder（天根公司），1~4分别为来源于LW-1、LW-C、LW-P和HL-1菌株样品。D. LW-C菌株携带BdCV1 dsRNA2全长序列的PCR扩增产物电泳图；M为DNA Marker II（天根公司），1~4分别为扩增dsRNA2全长的4个不同大小片段（1. dsRNA2-1；2. dsRNA2-2；3. dsRNA2-3；4. dsRNA2-4）的PCR扩增产物电泳图。

A. dsRNA detection by 1.2% agarose gel electrophoresis; M. DNA marker III (TIANGEN), 1 and 2. dsRNA derived from LW-1 and LW-C strains, respectively. B and C. RT-PCR detection for *RdRp* gene from BdPV1 and BdCV1, respectively; M. DNA 100 bp ladder (TIANGEN), 1-4. LW-1, LW-C, LW-P and HL-1 strains, respectively. D. RT-PCR detection for the full-length dsRNA2 from LW-C; M. DNA Marker II (TIANGEN), 1-4. The RT-PCR detection for 4 amplified segments from dsRNA2 of LW-C strain.

图1 LW-C携带BdCV1的dsRNA和RT-PCR检测电泳分析

Fig. 1 dsRNA and RT-PCR detection for BdCV1 infection with LW-C by 1.2% agarose gel electrophoresis

2.2 LW-C菌株的生物学培养特性和致病性测定

以LW-1、LW-P以及不带病毒的HL-1作为对照菌株，测定并分析25℃、黑暗培养3 d后LW-C菌落形态、菌株的生长速度以及致病性；评价其单独感染BdCV1对梨轮纹病菌菌株的生物学特性影响。结果表明，LW-C生长缓慢，甚至在培养基上停止生

长,菌落畸形,菌落边缘不规则,有扇变现象,色素产生异常;平均生长速率为4.29 mm·d^{-1},难产孢,与LW-1菌株的生长特性无显著性差异(图2-A、B)。对照组LW-P和'华梨1号'(HL-1)菌落气生菌丝多,后期产生灰绿色色素,菌株在PDA上生长迅速,3 d长满整个PDA平板;LW-P和HL-1的平均生长速率分别为22.05和15.5 mm·d^{-1}(图2-A、B)。

采用力度一致的3针针刺法接种离体'黄冠'梨果实,测定其致病性,结果表明:25 ℃保湿接种梨果7 d,空白对照未发病,LW-1和LW-C菌株引起的梨果病斑平均直径分别为4.45和4.63 mm;对照组LW-P和HL-1均可以在梨果上形成明显轮纹状病斑,病斑平均直径分别为38.65 mm和34.56 mm(图2-C、D)。因此,LW-C在生长速度以及致病性测定结果均与LW-1测定结果无显著性差异,2者生物学培养特性一致。

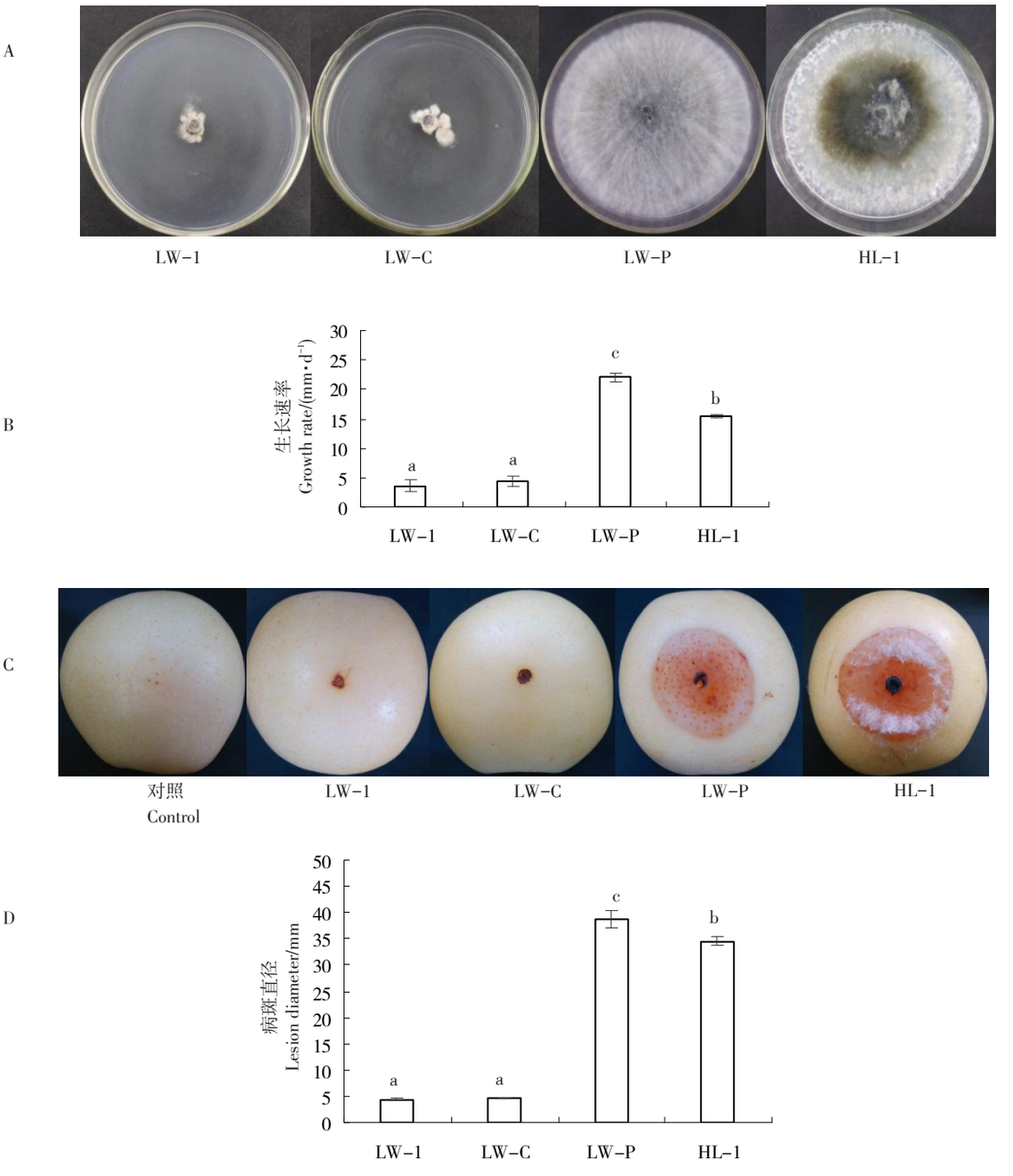
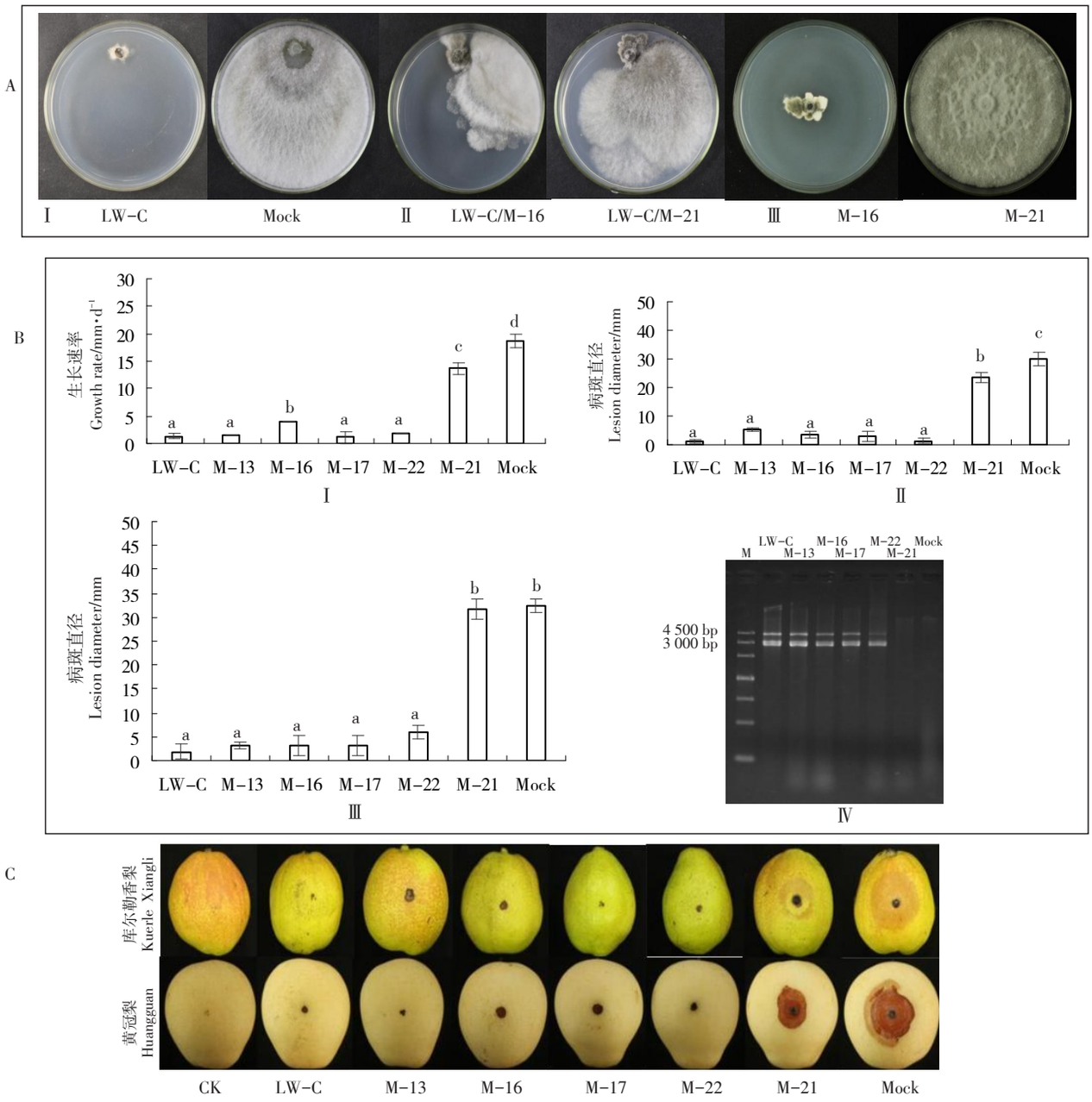


图2　LW-1、LW-C、LW-P及HL-1的菌落形态(A)、生长速度(B)、发病情况(C)及病斑直径(D)

Fig. 2　Colony morphologies (A), growth rate (B) on PDA, lesion length (C), and pathogenicity tests (D) on 'Huangguan' pear fruits for LW-1, LW-C, LW-P and HL-1 of *B. dothdiea* strains

2.3 LW-C菌株传毒特性的测定

在接种单一菌株的培养皿中，Mock生长速度很快，3 d就长满整个培养皿，而LW-C生长较慢，约占培养皿1/10（图3-A，Ⅰ）。在对峙培养处理中Mock前2 d生长十分迅速，菌丝接触之后生长受到抑制，出现畸形的菌落形态，如LW-C/M-16（图3-A，Ⅱ）；



A. LW-C 向强毒菌株 Mock 的传染；I 为 LW-C 和 Mock 菌株培养 3 d 的菌落形态；II 为 LW-C 和 Mock 对峙培养 3 d 菌株形态；III 为对峙培养衍生菌株 M-13 和 M-21 培养 7 d 菌落形态。B. I 为水平传染后衍生菌株的生长速度，II 和 III 分别为库尔勒香梨和黄冠梨果实病斑直径，IV 为衍生菌株 dsRNA 琼脂糖凝胶电泳检测。C. 水平传染后衍生菌株在分别接种在库尔勒香梨 5 d 和黄冠梨果实 8 d 的发病情况；CK 为空白对照在梨果上的发病情况。

A. The horizontal transmission of dsRNA with LW-C to Mock. Colony morphologies of LW-C and Mock cultured on PDA at 3 d (I) and contact culture at 3 d (II); Culture morphologies of derivates of M-16 and M-21 at 7 d, derived from the colony margin of Mock strain (III). B. Growth rate (I) and lesion diameter of the Kuerle Xiangli (II) and Huangguan pear (III); dsRNA identification (IV) for derivates (M-13, M-16, M-17, M-22 and M-21 strains) from Mock, M. DNA marker III(TIANGEN). C. Pear lesion on Kuerle Xiangli and Huangguan pear caused by M-13, M-16, M-17, M-22 and M-21 of *B. dothdiea* strains, respectively.

图 3 LW-C 的弱毒特性以及 dsRNA 水平传染

Fig. 3 The hypovirulent characterization of the strain LW-C of *B. dothidea* and the horizontal transmission of dsRNA with LW-C to Mock

也有少量的发现基本没有抑制现象,如LW-C/M-21(图3-A,Ⅱ)。Mock与LW-C对峙培养处理中共随机挑选5个衍生菌株,分别命名为M-13、M-16、M-17、M-21和M-22,并作为研究对象进行分析,其中4个衍生菌株(M-13、M-16、M-17和M-22)中表现出与亲本Mock不同的培养性状,菌落出现了与LW-C菌落表现一致的扇变区域,生长速度变慢,亲本菌株平均生长速率由18.5 mm·d^{-1}减至1.1~3.9 mm·d^{-1}(M-13、M-16、M-17和M-22平均生长速度分别为1.5、3.9、1.1和1.9 mm·d^{-1})(图3-B,Ⅰ);同时接种离体梨果实,致病性测定结果表明,衍生菌株分别在接种5 d'库尔勒香梨'果实上引起的病斑直径为1.3~5.1 mm(M-13、M-16、M-17和M-22平均病斑直径分别为5.1、3.4、2.8和1.3 mm),亲本病斑直径为29 .7mm(图3-B、C)。衍生菌株接种8 d'黄冠'梨果实上引起的病斑直径为3.1~5.9 mm(M-13、M-16、M-17和M-22平均病斑直径分别为3.2、3.1、3.1和5.9 mm),亲本接种'黄冠'梨果病斑直径为32 mm(图3-B、C)。4个衍生菌株的菌丝中都检测到了与LW-C携带BdCV1的4条dsRNA带型一致的dsRNA特征条带(图3-B,Ⅳ)。

另外,笔者也发现对峙培养衍生菌株中也获得1个菌株M-21,电泳检测无任何dsRNA特征条带(图3-B,Ⅳ);对其菌落形态及致病性测定,均表明M-21与亲本菌株表现出一致的性状,培养7 d,M-21产生大量分生孢子器(图3-A,Ⅲ),平均生长速度为13.6 mm·d^{-1};在梨果实上接种'库尔勒香梨'5 d,产生直径为23.6 mm的水渍状病斑(图3-B,Ⅱ和图3-C);接种'黄冠'梨8 d产生直径为31.6 mm的轮纹状病斑,与亲本菌株产生病斑状况无明显差异,表明LW-C携带的BdCV1没有传入Mock亲本菌株(图3-B,Ⅲ和图3-C);以上结果明确了LW-C携带的BdCV1 4条dsRNA片段的传染特性及其水平传染Mock后获得的衍生菌株的生物学特性和致病性。

3　讨　论

梨轮纹病是由葡萄座腔菌[*Botryosphaeria dothidea* (Moug:Fr) Ces & De Not]引起,通常在梨园中与梨树干腐病和梨树腐烂病复合感染,被称为梨树枝干3大病害,导致树势早衰,造成大量死树和毁园,给梨产业带来严重的经济损失[18-20]。梨轮纹病危害逐年加重,寻找新的、安全有效的措施防治梨轮纹病显得尤为迫切,弱毒相关病毒导致病原真菌致病力衰退,可作为真菌病害生防的一条重要途径,如板栗疫病和油菜菌核病的防治,均为真菌病毒用于真菌病害防治的成功案例[10-12,15]。研究报道显示,产黄青霉病毒科成员病毒可侵染一些真菌,但对植物病原真菌寄主的表型几乎没有抑制作用,未引起寄主生长发育异常以及致病力出现衰退的生物学特性变化[13,21-26]。本研究从前期获得LW-1菌株经多代纯化,经dsRNA和RT-PCR检测确认LW-1中分离获得仅含BdCV1分离株,命名为LW-C。分析其LW-C菌株生物学特性发现,强致病力菌株LW-P和HL-1菌株在PDA上菌落为白色,后期变为灰绿色;菌株生长速率快,培养3 d几乎长满整个平板,而LW-C分离株表现出生长缓慢,易产色素,难产孢,菌落畸形、边缘不规则、扇变现象,与复合感染BdCV1和BdPV1的衰退菌株LW-1一致。对其致病力测定表明,25 ℃、针刺接种'黄冠'梨果实7 d,强致病力对照菌株HL-1和LW-P均在梨果上造成明显轮纹病斑,平均直径为34 mm和38 mm;相比之下,LW-C菌株在梨果上发病病斑仅为4.6 mm,与LW-1菌株表现出一致的弱致病力甚至不致病。对其进行水平传毒,结果表明,对峙培养过程中明确可以观察到LW-C对Mock强致病力菌株具有抑制作用,后代表现出衰退、弱致病力的生物学特性;评价LW-C菌株对其他地理来源不同、遗传背景不同的梨轮纹菌株传毒效果,需进一步研究。以上研究结果直接证明BdCV1因子对寄主菌落形态、生长速度有抑制作用,导致寄主弱致病力;这是首次证实产黄青霉病毒科病毒可直接导致葡萄座腔菌菌株衰退,引起寄主致病力衰退,具有弱毒特性的研究。由于梨树抗病品种缺乏、病原菌传播容易及其寄主范围广泛等原因,轮纹病的防治一直是研究的难点。在梨树产业受真菌病害危害严重而又未能找到有效解决途径的情况下,在其致病菌弱毒株中发现dsRNA病毒具有较大的潜在意义[14]。因此,真菌病毒BdCV1是一种独特的病毒,为*B. dothdiea*真菌引起的梨、苹果等果树的轮纹病害的防治及其病原菌分子生物学特性研究提供了新颖的材料。真菌病毒生防应用的主要限制因素是菌株间的营养不亲和性限制真菌病毒传播,因此,进一步测定其传染能力,评价是否能够水平转染果树轮纹病菌以及其他不同种类真菌,或尝试BdCV1病毒粒体体外侵染轮纹病菌能力,评价对轮

纹病的防治作用，深入挖掘BdCV1的生防潜力。

前期研究证实产黄青霉病毒BdCV1基因组鉴定为4条dsRNA基因组片段，编码4个蛋白，其中2个蛋白为RdRp和CP，另外编码的2个蛋白功能研究尚属空白[13-14,21-26]，BdCV1对寄主有显著的影响，推定与其编码的4个蛋白功能有关，产黄青霉病毒科病毒蛋白在真菌致病性及与寄主互作中的作用等功能研究尚属空白。作为研究丝状真菌与病毒互作模式系统的板栗疫菌（*Cryphonectria parasitica*）感染低毒病毒CHV1（*Cryphonectria Hypovirus 1*）后，导致板栗疫病弱毒力作用机制涉及几个方面的因素，如低毒病毒对寄主板栗疫菌基因表达有影响，这些基因涉及与寄主生长、发育、致病等生物学特性以及病毒在寄主细胞内的复制和增殖等生命活动所需寄主因子，低毒病毒对板栗疫病菌的信号转导途径以及寄主致病基因和代谢途径等均有影响[27-31]。Li等[32]报道弱毒特性相关的SsDRV诱导的核盘菌下调表达基因与核盘菌生长发育异常及弱致病力有关，采用正反遗传方法，已证实1个编码*S. sclerotiorum* integrin-like（*SSITL*）蛋白具有影响核盘菌生长和致病性的功能[33-34]。进一步研究介导梨轮纹病菌毒力衰退相关真菌病毒与寄主互作，从转录和蛋白水平上分析病毒与寄主互作关系，明确BdCV1基因组中引起梨轮纹病菌致病力衰退的关键基因及梨轮纹病菌应答病毒侵染的分子机制，旨在获得与病毒互作和梨轮纹病菌致病相关的关键基因和信号转导途径，揭示真菌病毒引起寄主衰退的分子机制和轮纹病菌的致病机制，为果树轮纹病害的防治提供分子信息和基因资源。

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