

# 红灯樱桃采后主要病原真菌的鉴定及 *Bacillus velezensis* G-1 天然产物的广谱抑菌效果

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**摘要:**【目的】明确 *Bacillus velezensis* G-1 天然产物在樱桃采后抗真菌病害中的抑菌效果,为樱桃采后病害广谱生物防治药剂研发提供新型开发源。【方法】首先,以红灯樱桃采后病果为材料,通过组织块分离、纯化、形态特征、ITS 序列鉴定、特异基因和科赫法则确定樱桃采后病原真菌种类;其次,以获得的病原菌为靶标,通过生长速率法和平板对扣法研究了 *B. velezensis* G-1 脂肽和 2,4-二叔丁基苯酚的广谱抑菌效果。【结果】米根霉 YT-1(*Rhizopus oryzae*)、镰刀菌 YT-2(*Fusarium dlamini*)、藤仓镰刀菌 YT-4(*Fusarium fujikuroi*)、链格孢属 YT-5 与 YT-7(*Alternaria* sp.)、天竺葵葡萄孢 YT-6(*Botrytis pelargonii*)、冻土毛霉 YT-8(*Mucor hiemalis* f. *hiemalis*)和皮壳青霉 YT-9(*Penicillium crustosum*)为红灯樱桃采后主要病原真菌;8 株病原真菌中以菌株 YT-1、YT-6 和 YT-8 致病性较强; *B. velezensis* G-1 脂肽物质(500  $\mu\text{g} \cdot \text{mL}^{-1}$ )除对 *M. hiemalis* f. *hiemalis* 和 *P. crustosum* 的抑菌率低于 60%外,对其余各病原真菌抑菌率均高于 70%;100  $\mu\text{L}$  挥发性物质 2,4-二叔丁基苯酚(10  $\mu\text{g} \cdot \text{mL}^{-1}$ )除对 *P. crustosum* 的抑菌率为 0.41%外,对其余各病原真菌的抑菌率均高于 90%。【结论】首次在红灯樱桃中发现了镰刀菌(*F. dlamini*)和藤仓镰刀菌(*F. fujikuroi*),说明近年来红灯樱桃采后病害发生了变化,新增了镰刀菌属病原真菌;发现 *B. velezensis* G-1 脂肽物质和挥发性物质 2,4-二叔丁基苯酚可用于防治樱桃上述采后病害。

**关键词:**红灯樱桃;采后真菌病害;形态鉴定;ITS 序列鉴定;科赫法则;广谱抑菌

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## Identification of main postharvest pathogenic fungi in Hongdeng cherry and the anti-fungal spectrum of *Bacillus velezensis* G-1 natural product

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**Abstract:**【Objective】Postharvest fungal disease is one of the important factors affecting storage of sweet cherry [*Cerasus avium* (L.) Moench], and mycotoxins produced by pathogenic fungi can contaminate sweet cherry. In addition, chemical pesticides have been forbidden to be used during postharvest storage in many countries. On the other hand, some new pathogens have been founded from many crops in recent years. Therefore, it is important to clarify whether there are new diseases in postharvest sweet cherry fruits for the management of pre-harvest diseases and the technological innovation of post-harvest control. Our study aimed to explore the potential application of natural products from *Bacillus velezensis* G-1 to postharvest fungal diseases of sweet cherry in order to provide a new source for the development of broad-spectrum biocontrol agents for cherry postharvest diseases.【Methods】The fruits of Hongdeng sweet cherry infested, with postharvest diseases were used as materials to isolate the pathogenic fungi by the methods of tissue separation. The fruits were soaked in 75% ethanol for 30 s and rinsed three times by sterile distilled water. Then the fruits were soaked in 3% ethanol for 3 min and

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rinsed three times by sterile distilled water. The fruits were cut to 3 mm × 3 mm in size and plated on potato dextrose agar (PDA). All isolates were purified by single spore isolation method. All pure fungal isolates were identified by morphology and ITS sequence analysis. The morphology of the colony, spores size, mycelium and spore microscopy on PDA medium were used by morphology analysis. The pathogenic ability of the pathogenic fungi was tested based on Robert Koch's rule. In addition, the TEF (translation elongation factor-1 $\alpha$ ) was used to identify strain YT-2 and YT-4. NCBI's BLASTn tools were used to obtain highly homologous DNA sequence in GenBank and molecular phylogenetic tree was constructed by MEGA 5.0. The growth rate method and plate-to-plate method were used to evaluate the antifungal spectrum of lipopeptide (500  $\mu\text{g} \cdot \text{mL}^{-1}$ ) and 2,4-Di-tert-butylphenol (10  $\mu\text{g} \cdot \mu\text{L}^{-1}$ ) produced by *B. velezensis* G-1 against the pathogenic fungi obtained as mentioned above.【Results】There were 8 types of pure fungi isolated from the disease symptomatic tissues, including YT-1 (*Rhizopus oryzae*), YT-2 (*Fusarium dlamini*), YT-4 (*Fusarium fujikuroi*), YT-5 and YT-7 (*Alternaria* sp.), YT-6 (*Botrytis pelargonii*), YT-8 (*Mucor hiemalis* f. *hiemalis*) and YT-9 (*Penicillium crustosum*). YT-1, YT-6 and YT-8 were the main pathogenic strains among the 8 strains, and had much higher pathogenic ability than other pathogenic fungi. TEF gene could be used to identify *F. dlamini* and *F. fujikuroi*. The antifungal rate of lipopeptide (500  $\mu\text{g} \cdot \text{mL}^{-1}$ ), produced by *B. velezensis* G-1, against *M. hiemalis* f. *hiemalis* and *P. crustosum* were less than 60%. However, the antifungal rates of lipopeptide against other fungi species were over 80%. In addition, the volatile compound 2, 4-di-tert-butylphenol (10  $\mu\text{g} \cdot \mu\text{L}^{-1}$ ) had much lower antifungal activity against *P. crustosum*, and the inhibition rate was only 0.41%. But the antifungal rates of 2, 4-di-tert-butylphenol against other pathogenic fungi were all over 90%.【Conclusion】In this study, *F. dlamini* and *F. fujikuroi* were found in Hongdeng cherry for the first time, indicating that the postharvest diseases of Hongdeng cherry have changed in recent years. Toxin contamination from *F. dlamini* and *F. fujikuroi* should be paid attention to during cherry storage. The lipopeptide and volatile 2,4-di-tert-butylphenol from *B. velezensis* G-1 could be potentially used to control postharvest diseases. But the final control effect of lipopeptide and 2, 4-di-tert-butylphenol still needs to verify in commercial storage conditions. This study would provide a theoretical basis for controlling postharvest fungal disease and for preventing pre-harvest fungal disease of Hongdeng sweet cherry in the future.

**Key words:** Hongdeng cherry; Postharvest fungal disease; Morphological identification; ITS sequence identification; Koch postulates; Antifungal spectrum

红灯樱桃(*Cerasus avium*(L.)Moench)是大连市农业科学研究院培育的我国自主选育品种,外观鲜艳、口感鲜美,富含花青素、多酚、胡萝卜素、维生素A和Fe等营养物质,深受消费者喜爱<sup>[1-4]</sup>。然而该品种呼吸强度高、肉质较软、皮薄易发生机械损伤、遇雨季易裂果、易发生霉变、不耐贮运,且生产中配套的采后病害防治和贮藏技术水平落后,导致其冷藏、贮藏和货架期间易发生品质裂变和暴发真菌病害而失去商品价值,造成巨大经济损失,在一定程度上限制了该品种的推广种植<sup>[5-7]</sup>。另外随着真菌毒素研究的深入,发现多数病原菌可产生危害人体健康的有害毒素。为保障食品安全,目前多数国家将果蔬真菌毒素含量纳入检测范围<sup>[8-9]</sup>,因此红灯樱桃采

后真菌病害防治对其采后降损和降低毒素含量超标风险具有重要意义。

近年来,为有效解决红灯樱桃和其他品种采后病害发生问题,研究者不仅发现了青霉属(*Penicillium* sp.)、灰葡萄孢(*Botrytis cinerea*)、链格孢属(*Alternaria* sp.)、褐腐病菌(*Monilinia laxa*)、新西兰匍柄霉(*Stemphylium eturmiunum*)、胶孢炭疽菌(*Colletotrichum gloeosporioides*)、匍枝根霉(*Rhizopus stolonifer*)和枝孢霉(*Cladosporium* spp.)等樱桃采后致病菌<sup>[6, 10-20]</sup>,而且发现使用植物精油<sup>[2, 15]</sup>、壳聚糖<sup>[12]</sup>、芽孢杆菌<sup>[19]</sup>、拮抗酵母<sup>[21-22]</sup>、气调保鲜<sup>[11]</sup>、紫外照射<sup>[23]</sup>、1-甲基环丙烯(1-MCP)<sup>[17]</sup>和采前+采后化学处理<sup>[12]</sup>等单一处理方式或组合处理可有效抑制其中1种或多

种病原引起的樱桃采后病害。这些研究在一定程度上明确了红灯樱桃及其他品种采后病害种类,降低了樱桃采后病害引起的经济损失,延长了贮藏时间和货架供应时间,但化学防治中不合理使用引起的农药残留超标和病原抗药性增强问题,物理防治措施的高成本问题以及不同产地、品种、栽培条件和种植年限下的采前、采后病原微生物种类差异和种群变化现象亦不可忽视<sup>[13,24-29]</sup>。此外,目前现有研究都还主要集中在已发现的共性病原防治技术开发或单一病原分离鉴定方面<sup>[6,10,17,19-20]</sup>,红灯樱桃采后贮藏期间是否还存在其他腐烂致病菌,近年来致病菌种类是否发生变化还不清楚,广谱抑菌天然产物筛选研究还较为匮乏。因此有关红灯樱桃采后病害种类的实时监测、鉴定、新病害预警以及配套广谱生物防治技术开发不仅对我国自主选育品种红灯樱桃采后病害针对性防治具有重要意义,而且对其采前病害管理、推广种植和品种保护具有重要意义。

经前期研究发现,*Bacillus velezensis* G-1生防菌可产生脂肽类和挥发性2,4-二叔丁苯酚物质(待发表),且已有研究发现这2类物质具有较好抗真菌谱<sup>[30-31]</sup>,在植物病害生物防治中具有较好的应用潜力,但有关这2类物质在樱桃采后病害广谱抑菌中的研究还较为匮乏。因此,基于上述研究背景,笔者在本研究中重点对红灯樱桃采后贮藏期的病原真菌种类和*B. velezensis* G-1天然产物的广谱抑菌效果进行研究,一方面旨在明确红灯樱桃采后贮藏期间的真菌病害种类,为后期针对性防治技术开发提供参考依据,降低红灯樱桃采后损失和毒素污染风险,提高食品安全性,延长销售时间,并为其采前病害管理提供警示性信息;另一方面为红灯樱桃采后真菌病害生物防治提供参考。

## 1 材料和方法

### 1.1 供试材料

1.1.1 樱桃材料 鲜红色红灯樱桃(*Cerasus pseudocerasus*)采自山西省绛县史村樱桃种植园区,采用泡沫冰盒运至实验室,剔除机械伤、病虫果,每盒4 kg,于0 ℃贮藏45 d后挑选不同症状病果用于病害分离。

1.1.2 拮抗细菌 菌株G-1分离自梓树果实,并经全基因组测序后鉴定为*Bacillus velezensis*(待发表),由山西省农业科学院农产品贮藏保鲜研究所采

后病理室采用20%(φ)甘油-80 ℃保藏。

### 1.2 供试培养基

PDA培养基:去皮马铃薯200 g、葡萄糖20 g、琼脂粉18 g、蒸馏水1000 mL;PDB培养液配方为PDA培养基去掉琼脂。采后病原真菌分离培养基为含硫酸链霉素终质量浓度100 μg·mL<sup>-1</sup>的PDA培养基。

### 1.3 甜樱桃采后病原真菌的分离与纯化

参照高振峰等<sup>[32]</sup>描述的消毒程序和分离、纯化方法略作修改,对甜樱桃贮藏期间采后病果中的病原真菌进行分离和纯化,具体程序为:①表面消毒(75%乙醇浸泡30 s,无菌水冲洗3次;3%次氯酸钠浸泡3 min,无菌水冲洗3次);②使用手术刀从病果病健交界处切取大小为3 mm×3 mm左右的组织块,并用接种针移至PDA平板,置于26 ℃恒温培养;③待菌丝长出后使用接种针挑取尖端菌丝或最外侧较纯菌丝置于装有500 μL无菌水的1 mL离心管中,震荡制备悬浮液,随后吸取80 μL菌悬液涂布于PDA平板,进行单孢分离纯化;④从单孢分离纯化平板上挑取纯菌落转至新PDA平板上进行二次纯化,待其长满整个平板且形态一致后,置于4 ℃保存备用。

### 1.4 樱桃采后病原真菌形态鉴定

参照赵倩<sup>[33]</sup>描述的方法略作修改:使用接种针将直径为5 mm的纯培养物菌饼接种至PDA平板中央,于26 ℃恒温培养7 d,期间每隔1 d进行菌落观察,并记录其形态特征和测量菌落直径(连续测量4 d),每组3次重复;培养7 d后从PDA平板上挑取菌丝或分生孢子制片,并用显微镜观察菌丝和分生孢子形态。每玻片至少观察20个视野,并选择典型特征视野对菌丝和孢子进行拍照和特征描述,3次重复。

### 1.5 樱桃采后病原真菌的ITS序列鉴定

1.5.1 发酵培养 采用接种针将直径为5 mm的各真菌纯培养物菌饼接种至150 mL无菌PDB培养液中,于26 ℃、160 r·min<sup>-1</sup>下恒温震荡培养7 d。发酵终止后于4 ℃、12 000 r·min<sup>-1</sup>离心10 min收集菌体,无菌水冲洗3次,用无菌滤纸吸干表面水分后经液氮研磨成粉,并于-80 ℃保存,用于后期基因组DNA提取。

1.5.2 基因组DNA提取 参照李焕宇等<sup>[34]</sup>的CTAB法提取1.5.1中收集的各真菌纯培养物基因组DNA。

1.5.3 ITS序列扩增与测序 除菌株YT-1和YT-5使用真菌通用引物对ITS1(5'-TCCGTAGGT-GAACCTGCGG-3')和ITS2(5'-GCTGC-GTTCTTCATCGATGC-3')进行扩增外,其余菌株均采用ITS1(5'-TCCGTAGGTGAACCTGCGG-3')和ITS4(5'-TCCTCCGCTTATTGATATGC-3')进行扩增。25 μL扩增体系:Premix Taq<sup>TM</sup>(TaKaRa Taq<sup>TM</sup> Version 2.0)12.5 μL;引物ITS1和ITS2各0.5 μL;基因组DNA 0.5 μL;ddH<sub>2</sub>O 11 μL。扩增程序:94 °C变性5 min;94 °C 45 s,55 °C 40 s,72 °C 30 s,30个循环;72 °C延伸10 min。PCR产物经电泳检测合格后送至北京六合华大基因测序。

1.5.4 菌株YT-2和YT-4的TEF基因扩增与测序 利用特异基因TEF(translation elongation factor-1α)引物EF1(5'-ATGGGTAAGGAGGACAAGAC-3')和EF2(5'-GGAGGTACCAAGTGATCATGTT-3')<sup>[35]</sup>,以菌株YT-2和YT-4基因组DNA为模板,分别对2个菌株的TEF基因进行扩增。25 μL扩增体系与ITS序列扩增体系相同。扩增程序:94 °C变性5 min;94 °C 50 s,60 °C 60 s,72 °C 40 s,30个循环;72 °C延伸10 min。PCR产物电泳检测合格后送至北京六合华大基因测序。

1.5.5 序列比对及NJ系统发育树构建 获得各菌株ITS序列和镰刀菌TEF序列后,首先使用NCBI在线blast软件同模式菌株进行序列比对;随后选取各菌株近缘模式菌的ITS或TEF基因序列,使用MEGA 5.0软件构建NJ系统发育树,从而确定各菌株分类地位。

### 1.6 樱桃采后病原真菌致病性回接验证

依据科赫法则,参照赵远征等<sup>[36]</sup>的描述略作修改后对各病原真菌致病性进行回接验证。首先使用接种针对经表面消毒(75%乙醇浸泡60 s)后的樱桃果实(鲜红色)进行针刺造成微伤口,每果实1个直径约3 mm的近圆形伤口;其次挑取活化培养7 d后的纯培养物,使用无菌水制备成孢子浓度为2×10<sup>6</sup> cfu·mL<sup>-1</sup>的悬浊液,取20 μL接种于伤口处;最后将樱桃果实置于90 mm培养皿,于装有无菌吸水棉球的PE保鲜袋中20 °C恒温保湿培养7 d,观察和记录发病情况,并对发病果实中的病原进行再次分离、纯化、鉴定(二次分离纯培养物与原分离物对比鉴定)。以20 μL无菌水作为对照,每处理3次重复,每重复3个果实。

### 1.7 *Bacillus velezensis* G-1天然产物广谱抑菌效果测定

1.7.1 脂肽物质抗真菌谱测定 参照高振峰<sup>[30]</sup>描述的盐酸沉淀、甲醇抽提的方法和生长速率法分别进行菌株G-1脂肽物质的提取和抑菌活性测定。脂肽物质质量浓度为500 μg·mL<sup>-1</sup>,以无菌水为对照,各处理和试验均3次重复。

1.7.2 挥发性物质2,4-二叔丁基苯酚抗菌谱测定 前期通过SPME-GC-MS检测发现*B. velezensis* G-1可产生2,4-二叔丁基苯酚,且已有研究发现2,4-二叔丁基苯酚对多种病原具有良好抑菌效果<sup>[34]</sup>。称取2,4-二叔丁基苯酚[阿拉丁(上海)]1 g,使用1 mL无水乙醇溶解后,取100 μL参照Gao等<sup>[31]</sup>描述的平板对扣法测定该物质对樱桃采后病原真菌的抑菌谱,以等体积(100 μL)无水乙醇为对照,各处理和试验3次重复。

### 1.8 数据处理

使用Excel 2010统计数据,使用Adobe Photoshop CS 6.0软件编辑图片,利用SPSS17.0软件进行Duncan's新复极差分析(*p* < 0.05)。

## 2 结果与分析

### 2.1 甜樱桃采后病原真菌分离、纯化结果及形态特征

采用组织块分离和单孢纯化法,从红灯樱桃病健交界处组织中共分离、纯化出9种形态差异明显的纯培养物,编号为YT-1~9。9种纯培养物中以菌株YT-1生长最快,其次为YT-8和YT-6,YT-3生长速率最慢。在菌落、菌丝和孢子形态特征方面,YT-1同根霉属(*Rhizopus* sp.)较为相似;YT-2和YT-4同镰刀菌(*Fusarium*)较为相似;YT-3形态特征变化较为明显,具有典型的酵母和真菌形态特性,同短梗霉属(*Aureobasidium* sp.)较为相似;YT-5和YT-7同链格孢属(*Alternaria* sp.)较为相似,其中YT-5和YT-7除PDA平板菌落形态存在一定差异外,菌丝和孢子形态均极为接近;YT-6同葡萄孢属(*Botrytis* sp.)较为相似;YT-8同毛霉属(*Mucor* sp.)较为相似;YT-9同青霉属(*Penicillium* sp.)较为相似(表1)。

### 2.2 甜樱桃采后病原真菌的分子生物学鉴定

使用ITS通用引物对9种真菌基因组DNA扩增后发现,引物ITS1和ITS2可对菌株YT-1和YT-5 ITS有效扩增,而引物ITS1和ITS4可对其余菌株

表 1 9 种真菌的 PDA 平板菌落、菌丝和孢子形态特征

Table 1 Morphological characteristic of colonies, mycelium and spores of nine fungi on PDA plate

菌株编号 Strain number	形态特征 Morphological characteristic
YT-1	<p>平板形态: 菌丝生长速率 <math>2.83 \text{ cm} \cdot \text{d}^{-1}</math>; 生产初期菌丝呈白色, 基内菌丝明显; 生长后期产生气生菌丝, 菌丝颜色变为灰褐色且菌丝末端产生大量黑色孢子。</p> <p>显微形态: 菌丝无隔、褐色; 有明显孢子囊, 孢囊梗直立或稍弯曲, 孢子囊呈球形或近球形。孢子多呈椭圆形, 与杏仁相似, 表面有条形纹路, 大小范围 <math>(10.73\text{--}23.53)\mu\text{m} \times (10.25\text{--}18.97)\mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>2.83 \text{ cm} \cdot \text{d}^{-1}</math>. Strain YT-1 could produce intrabasal mycelium and aerial mycelium, and the color of mycelium changed from white to gray-brown with growing. In addition, a large number of black spores were produced from mycelium in the late stage mycelium.</p> <p>Microscopic morphology: The mycelium was brown and no septum. Obvious sporangia could be produced in the top of mycelium and the sporangiophore was upright or slightly curved. Besides, the sporangia was spherical or nearly spherical. The spores shape were oval similar to almonds, and had striped lines on the surface. The size of the spores were <math>(10.73\text{--}23.53)\mu\text{m} \times (10.25\text{--}18.97)\mu\text{m}</math>.</p>
YT-2	<p>平板形态: 菌丝生长速率 <math>0.90 \text{ cm} \cdot \text{d}^{-1}</math>; 生长初期有明显基内菌丝, 菌丝白色, 后期产生淡紫色色素, 且正面紫色不明显, 背面明显淡紫色, 培养基无色。</p> <p>显微形态: 菌丝无隔, 透明, 有明显分叉; 分生孢子似镰刀形, 略弯曲, 两端渐尖, 0~3 个分隔, 大小范围 <math>(9.02\text{--}13.97)\mu\text{m} \times (3.69\text{--}7.21)\mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>0.90 \text{ cm} \cdot \text{d}^{-1}</math>. In the early stage of growth strain YT-2 could produce white intrabasal mycelium, and lavender pigments were produced in the later stage. In addition, the purple on plate front was not obvious, but the back was obviously lavender.</p> <p>Microscopic morphology: The mycelium was no septum, transparent, but had obvious bifurcations. The spores were sickle-shaped, slightly curved, and gradually pointed at both ends. Conidias had 0-3 separations. The size of the spores were <math>(9.02\text{--}13.97)\mu\text{m} \times (3.69\text{--}7.21)\mu\text{m}</math>.</p>
YT-3	<p>平板形态: 菌丝生长速率 <math>0.33 \text{ cm} \cdot \text{d}^{-1}</math>; 菌落初期和后期形态变化明显; 菌落最初黏稠, 灰白色, 表面湿润, 同酵母形态类似; 后期菌落颜色有向黑色转变趋势, 且长出乳白色菌丝, 中心黑色, 同真菌形态类似。</p> <p>显微形态: 菌丝无隔膜; 分生孢子卵形, 大小范围 <math>(9.94\text{--}14.72)\mu\text{m} \times (4.08\text{--}5.69)\mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>0.33 \text{ cm} \cdot \text{d}^{-1}</math>. The initial colony characters of stain YT-3 includ viscous, grayish white, with a moist surface and similar to the shape of yeast. In the later stage, the colony color had a tendency to change to black, and milky white mycelium with a black center had been produced, which similar to the shape of fungi.</p> <p>Microscopic morphology: The mycelium was no septum. The spores morphology was ovoid, and with a size range of <math>(9.94\text{--}14.72)\mu\text{m} \times (4.08\text{--}5.69)\mu\text{m}</math>.</p>
YT-4	<p>平板形态: 菌丝生长速率 <math>0.85 \text{ cm} \cdot \text{d}^{-1}</math>; 菌落表面有棉絮状或毛毡状白色菌丝; 生长初期菌丝白色, 有明显基内菌丝, 后期产生紫色色素。</p> <p>显微形态: 菌丝无隔, 透明, 有分叉; 分生孢子卵圆形、长椭圆形、无隔、大小范围 <math>(8.04\text{--}16.20)\mu\text{m} \times (4.12\text{--}5.73)\mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>0.85 \text{ cm} \cdot \text{d}^{-1}</math>. Cotton wool-like or felt-like white mycelium on the surface of the colony. In early growth, the strain YT-4 had obvious intrabasal and the mycelium colorwas white, but mycelium produced purple pigments in the later stage. The back of the medium was purple-black, and the medium was brown.</p> <p>Microscopic morphology: The mycelium was transparent, bifurcated, and no septum. The spores were ovoid or oblong and without septum. In addition, the size range of spores were <math>(8.04\text{--}16.20)\mu\text{m} \times (4.12\text{--}5.73)\mu\text{m}</math>.</p>
YT-5	<p>平板形态: 菌丝生长速率 <math>0.83 \text{ cm} \cdot \text{d}^{-1}</math>; 生长初期菌丝白色, 有明显基内菌丝; 生长后期, 菌丝转为墨绿色, 产生大量孢子。</p> <p>显微形态: 菌丝有隔膜; 分生孢子黄褐色, 大小范围 <math>(46.47\text{--}64.28)\mu\text{m} \times (14.68\text{--}17.46)\mu\text{m}</math>, 有 1~3 个横隔膜, 0~1 个纵隔膜, 无喙或具短柱状假喙。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>0.83 \text{ cm} \cdot \text{d}^{-1}</math>. In the early growth, strain YT-5 had obvious intrabasal mycelium, and the mycelium colorwas white. In the later stage, the color of the mycelium turns to dark green, producing a large number of spores, and the back of the plate was turned to dark black.</p> <p>Microscopic morphology: The mycelium had septums. The spore characters were include yellow-brown, with 1~3 diaphragms and 0~1 mediastinum, and no beak or short columnar false beak. In adition, the size range of spore was <math>(46.47\text{--}64.28)\mu\text{m} \times (14.68\text{--}17.46)\mu\text{m}</math>.</p>
YT-6	<p>平板形态: 菌丝生长速率 <math>1.38 \text{ cm} \cdot \text{d}^{-1}</math>; 初期菌丝疏松、白色、有基内菌丝, 匍匐状覆盖全培养皿; 后期菌丝颜色转为土灰色, 产生大量土黄色孢子。</p> <p>显微形态: 菌丝有隔膜、黄褐色; 分生孢子多为椭圆形、倒卵形、大小范围 <math>(22.14\text{--}27.85)\mu\text{m} \times (12.81\text{--}16.88)\mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>1.38 \text{ cm} \cdot \text{d}^{-1}</math>. The initial mycelium characters incloud loose, white color, with basal mycelium, the center mycelium was dense, growing as a radial shape, and creeping to cover the whole petri dish. In the later stage, the mycelium color turned to earthy gray and produced many earthy yellow spores.</p> <p>Microscopic morphology: The mycelium had septum and with yellowish brown color. The spores shapes were oval or obovate, and the size range was <math>(22.14\text{--}27.85)\mu\text{m} \times (12.81\text{--}16.88)\mu\text{m}</math>.</p>

表1(续)  
Table 1(Continued)

菌株编号 Strain number	形态特征 Morphological characteristic
YT-7	<p>平板形态:菌丝生长速率<math>0.85 \text{ cm} \cdot \text{d}^{-1}</math>;生长初期菌丝白色,边缘整齐,随后产生土色色素,后期菌丝颜色变为土灰色;菌落平展,絮状,疏松,背面黑色。</p> <p>显微形态:菌丝有隔膜、黄褐色、有分枝;分生孢子黄褐色,大小范围<math>(48.13\sim53.16) \mu\text{m} \times (18.07\sim19.39) \mu\text{m}</math>,有1~4个横膈膜,无喙或具短柱状假喙。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>0.85 \text{ cm} \cdot \text{d}^{-1}</math>. In the early growth, the mycelium color was white and had neat edges. In the later stage, earth-colored pigments were produced, and the color of mycelium turned to earthy gray. Besides, the colonies were flat, flocculent, loose, and the backside was black.</p> <p>Microscopic morphology: The mycelium had the characters of yellowish-brown, with septum and branched. The spores were yellowish-brown color, with a size range of <math>(48.13\sim53.16) \mu\text{m} \times (18.07\sim19.39) \mu\text{m}</math>, with 1~4 diaphragms, and no beak or short columar pseudobea.</p>
YT-8	<p>平板形态:菌丝生长速率<math>1.40 \text{ cm} \cdot \text{d}^{-1}</math>;生长初期菌丝白色,后期转为灰色,有棉质感。可产生有灰褐色假根的气生匍匐菌丝。</p> <p>显微形态:菌丝无隔膜、透明;孢子卵圆形或球形、无色、表面平滑,大小范围<math>(26.14\sim30.23) \mu\text{m} \times (10.61\sim12.48) \mu\text{m}</math>。</p> <p>PDA plate morphology: Growth rate of mycelial was <math>1.40 \text{ cm} \cdot \text{d}^{-1}</math>. The mycelium color was white in the early stage of growth, but turned to gray in later stage, and with obvious cotton texture. Strain YT-8 had aerial creeping mycelium, and the creeping mycelium produced gray-brown pseudo-roots.</p> <p>Microscopic morphology: The mycelium was transparent and no septum. The spores were ovoid or spherical, colorless, and had a smooth surface. The size range of spore was <math>(26.14\sim30.23) \mu\text{m} \times (10.61\sim12.48) \mu\text{m}</math>.</p>
YT-9	<p>平板形态:单菌落很难覆满整个平板,生长初期菌落周围有白色较短绒毛,边缘整齐,后期产生大量灰绿色孢子,反面呈微黄褐色。</p> <p>显微形态:营养菌丝体无色、有横隔,光滑;分生孢子多为球形,光滑,大小范围<math>(6.80\sim8.25) \mu\text{m} \times (5.95\sim7.69) \mu\text{m}</math>。</p> <p>PDA plate morphology: It is difficult for a single colony of strain YT-9 to cover the entire plate on PDA plate. In the early growth, the colony was surrounded by short white hairs and had neat edges. In the later stage, a large number of gray-green spores were produced, and the back of the medium was yellowish brown.</p> <p>Microscopic morphology: The vegetative mycelium was colorless, and with septum. The spores were spherical, smooth, and with a size range of <math>(6.80\sim8.25) \mu\text{m} \times (5.95\sim7.69) \mu\text{m}</math>.</p>

有效扩增。扩增产物经华大基因测序后菌株YT-1~9的ITS序列长度分别为:269、534、554、530、244、526、543、624和562 bp。菌株YT-1~9 NCBI序列登录号分别为MT477703、MT477704、MT477705、MT477706、MT477707、MT477708、MT477709、MT477710和MT477711。

使用NCBI对各菌株ITS序列进行blast比对后,选取9种病原真菌及各近缘模式菌株ITS序列,并用MEGA 5.0软件构建NJ系统发育树。发现菌株YT-1 ITS序列同模式种*Rhizopus oryzae* CBS 11207 (JN206323.1)聚在同一支;菌株YT-2和YT-4均同模式种*Fusarium fujikuroi* CBS 221.76<sup>T</sup> (NR 111889.1)和*Fusarium dlamini* CBS 738.97<sup>T</sup> (MH862668.1)聚在同一支,说明2个菌株在ITS序列上具有较高的相似度,且均同2模式菌株具有较高同源率;菌株YT-3同模式种*Aureobasidium pullulans* CBS 584.75<sup>T</sup> (NR 144909.1)聚在同一支;菌株YT-5、YT-7均同模式种*Alternaria cerealis* CBS 119544<sup>T</sup> (NR 136117.1)、*Alternaria angustiovoidea* CBS 195.86<sup>T</sup> (MH861939.1)、*Alternaria doliconidium* HKAS 100840<sup>T</sup> (NR 158361.1)、*Alternaria alstroemeriae* CBS 118809<sup>T</sup>

(NR 163686.1)、*Alternaria doliconidium* KUMCC 17-0263<sup>T</sup> (MG828864.1)、*Alternaria beta-kenyensis* CBS 118810<sup>T</sup> (NR 136118.1)、*Alternaria arborescens* CBS 102605<sup>T</sup> (NR 135927.1)、*Alternaria destruens* ATCC 204363<sup>T</sup> (NR 137143.1)和*Alternaria iridialis* CBS 118486<sup>T</sup> (NR 136120.1)聚在同一支;菌株YT-6同模式种*Botrytis pelargonii* CBS 497.50<sup>T</sup> (NR 159600.1)聚在同一支;菌株YT-8同模式种*Mucor hiemalis* f. *hiemalis* CBS 201.65<sup>T</sup> (NR 152948.1)聚在同一支;菌株YT-9同模式菌株*Penicillium crustosum* CBS 115503<sup>T</sup> (MH862985.1)和*Penicillium crustosum* FRR 1669<sup>T</sup> (NR 077153.1)聚在同一支。

为进一步明确镰刀菌YT2和YT4的分类地位,引入特异基因TEF对2个菌株进行了二次鉴定。对菌株YT-2和YT-4的TEF基因PCR产物进行测序后获得的TEF基因序列长度分别为382 bp和340 bp,NCBI序列登录号分别为MW408701和MW408702。使用2个菌株及近缘种的TEF基因构建NJ系统发育树后发现,菌株YT-2和YT-4在聚类上存在明显差异,菌株YT-4同模式种*Fusarium fuj-*

*kuroi* B14<sup>T</sup> (FMSL01000004.1:1353628~1355389) 聚在单独一枝,且自展值为99,而菌株YT-2则同*Fusarium dlamini* NRRL 13164<sup>T</sup> (KU171721.1)聚在一起,自展值为96。对2个菌株同近缘种之间的遗传距离进行分析后同样发现菌株YT-4和YT-2分别同

*Fusarium fujikuroi* B14<sup>T</sup> (FMSL01000004.1:1353628~1355389) 和 *Fusarium dlamini* NRRL 13164<sup>T</sup> (KU171721.1) 的遗传距离最近,分别为0.000和0.007,且2个菌株之间的遗传距离为1.528,说明2个菌株之间存在显著差异(表2)。

表2 菌株 YT-2 和 YT-4 与同支近缘种之间的遗传距离

Table 2 The genetic distance between strains YT-2 and YT-4 and their close relative species

菌株 Strain	YT-4	<i>Fusarium fujikuroi</i> B14	YT-2	<i>Fusarium dlamini</i> NRRL 13164	<i>Fusarium acutatum</i> NRRL 13308
YT4	0.000		0.624	0.660	0.879
<i>Fusarium fujikuroi</i> B14	0.000		0.624	0.660	0.879
YT2	1.528	1.528		0.007	0.045
<i>Fusarium dlamini</i> NRRL 13164	1.565	1.565		0.007	0.047
<i>Fusarium acutatum</i> NRRL 13308	2.157	2.157		0.211	0.221

注:左下角为遗传距离,右上角为标准误差。

Note: Distance in lower left, standard error in upper right.

### 2.3 甜樱桃采后病原回接验证

为进一步确定9种纯培养物是否为樱桃采后致病菌,采用科赫法则对其致病性进行了回接验证,结果表明,YT-1、YT-2、YT-4、YT-5、YT-6、YT-7、YT-8和YT-9在樱桃果实上均表现出发病症状,其中以YT-1、

YT-6和YT-8腐烂症状最为明显,20℃下培养7d腐烂面积大于果实1/2表面积,且各病原二次分离和二次ITS序列鉴定结果同之前一致。9种病原菌中以菌株YT-1和YT-6危害最为严重,培养7d后病原菌菌丝几乎布满全果实,而菌株YT-3则未表现出发病症状(图1)。

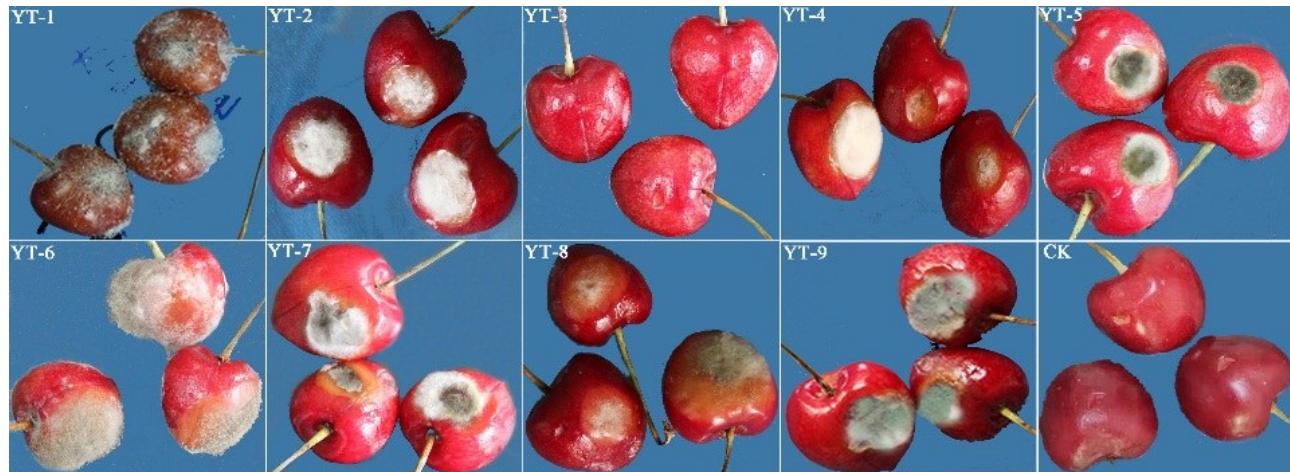


图1 人工接种病原菌7d后的发病症状

Fig. 1 Symptom after 7 days of cherry inoculated by pathogenic fungi

### 2.4 *Bacillus velezensis* G-1 抗菌物质对樱桃采后病原真菌抑菌效果

采用生长速率法和平板对扣法对 *B. velezensis* G-1 脂肽和挥发性抗菌物质 2,4-二叔丁基苯酚对樱桃采后8种病原真菌的抑菌效果进行测定。发现 *B. velezensis* G-1 脂肽物质对8种樱桃采后病原真菌具有良好的抑菌效果,其中对 YT-2、YT-4、YT-5 和 YT-

7 的抑菌效果较好,抑菌率均高于 80%;对 YT-1 和 YT-6 抑菌率可超过 70%,而对 YT-8 和 YT-9 抑菌效果则较差,抑菌率仅分别为 58.91% 和 58.59%(表3)。同脂肽物质相比,挥发性抗菌物质 2,4-二叔丁基苯酚除对 YT-9 菌株抑菌活性较差外(抑菌率 0.41%),对其余各菌株均具有较好抑菌活性,抑菌率均超过 95%(表3)。

表 3 *B. velezensis* G-1 脂肽和挥发性物质 2,4-二叔丁基苯酚对樱桃采后致病菌的抑菌效果Table 3 Antifungal effect of lipopeptide and 2, 4-Di-tert-butylphenol of *B. velezensis* G-1 on postharvest fungal disease of sweet cherry

病原真菌 Pathogenic fungi	脂肽物质抑菌效果 Antifungal activity of lipopeptide			2,4-二叔丁基苯酚抑菌效果 Antifungal activity of 2,4-Di-tert-butylphenol		
	对照病原菌落直径 Pathogenic colony diameter of control/mm	处理病原菌落直径 Pathogenic colony diameter of treatment/mm	抑制率 Inhibition rate/%	对照病原菌落直径 Pathogenic colony diameter of control/mm	处理病原菌落直径 Pathogenic colony diameter of treatment/mm	抑制率 Inhibition rate/%
	YT-1	85.33±0.58	26.17±0.76	73.65±1.10 c	85.33±0.58	5.33±0.58
YT-2	81.00±1.00	16.50±0.50	84.87±0.46 b	81.00±1.00	5.67±0.58	99.12±0.76 a
YT-4	81.17±0.76	13.00±1.32	89.51±1.64 a	81.83±0.76	6.00±1.00	98.71±1.29 a
YT-5	85.33±0.58	14.17±1.04	88.59±1.24 a	85.33±0.58	5.00±0.00	100.00±0.00 a
YT-6	85.67±0.58	25.17±1.04	75.00±1.35 c	85.67±0.58	5.67±0.58	99.18±0.71 a
YT-7	85.67±0.58	13.17±0.29	89.88±0.33 a	85.67±0.58	8.33±0.58	95.87±1.88 b
YT-8	85.33±0.58	38.00±1.32	58.91±1.93 d	85.33±0.58	5.67±0.58	99.17±0.72 a
YT-9	23.17±0.76	12.50±0.50	58.59±4.44 d	85.67±0.58	85.33±0.58	0.41±0.71 c

注:同列不同小写字母代表  $p < 0.05$  水平上差异显著。

Note: Different small letters in the same column represent significant differences at  $p < 0.05$ .

### 3 讨 论

采后病害作为影响果蔬采后产业链健康发展的关键因素之一,有效控制其发生对保持果蔬品质、降低真菌毒素污染风险、延长贮藏、货架和加工时间具有重要意义,特别是对降低易腐果蔬(如:草莓、树莓、桑葚、樱桃、番茄和葡萄等)采后损失意义重大<sup>[29, 37-39]</sup>。另外,还有研究发现,随着种植年限和环境变化会出现新病害<sup>[26-29]</sup>,因此为明确近年来山西史村红灯樱桃是否有新采后病害出现和有效控制其发生,笔者在本研究中首先对红灯樱桃采后病原真菌进行了分离与鉴定,并在此基础上探究了前期发现的 *B. velezensis* G-1 脂肽和挥发性抗菌物质 2,4-二叔丁基苯酚对其采后真菌病害的广谱抑菌效果,研究结果不仅明确了红灯樱桃采后病原真菌种类,而且发现了 *B. velezensis* G-1 脂肽和挥发性物质 2,4-二叔丁基苯酚在樱桃采后病害广谱防治的应用潜力,为樱桃采后病害生物防治、防腐保鲜技术和真菌毒素污染风险控制技术开发奠定了理论基础。

采用组织块分离法和单孢纯化相结合的方法从病果病、健交界处共分离和纯化出 9 种真菌纯培养物(编号为 YT-1~9)。菌株 YT-1 形态特征与已报道的龙牙百合种球腐烂的病原米根霉 ycxy-yb(*Rhizopus oryzae*)<sup>[40]</sup>和桑根腐病病原米根霉 TY.GF1(*Rhizopus oryzae*)<sup>[41]</sup>极为接近;菌株 YT-2 形态特征与 *Fusarium dlaminii* ZH-H2(CGMCC No.9316) 和 Marasas 等<sup>[42]</sup>及 Chehri 等<sup>[43]</sup>描述的 *Fusarium dlaminii* 形态

特征较为相似,而与侯恩庆<sup>[44]</sup>和施祖荣等<sup>[45]</sup>描述的藤仓镰刀菌(*F. Fujikuroi*)形态特征差别较大;菌株 YT-3 形态特征与已报道的 *Aureobasidium pullulans* A5 较为相似<sup>[46]</sup>;菌株 YT-4 形态特征与藤仓镰刀菌 GF1<sup>[44]</sup>较为相似;菌株 YT-5 和 YT-7 形态特征与赵倩<sup>[33]</sup>描述的多种链格孢病原较为相近;菌株 YT-6 形态特征与吴小瑶<sup>[47]</sup>描述的 *Botrytis pelargonii* 较为相近;菌株 YT-8 形态特征与 Magray 等<sup>[48]</sup>已报道的 *Mucor hiemalis* 较为相近;菌株 YT-9 形态特征与已报道的 *Penicillium crustosum* QH11<sup>[49]</sup>较为相近。采用形态鉴定、ITS 序列和特异基因 TEF(Translation elongation factor 1 alpha) 可将 YT-2 鉴定为镰刀菌(*F. dlaminii*), YT-4 鉴定为藤仓镰刀菌(*F. fujikuroi*),说明 TEF 基因在镰刀菌小种鉴定上具有一定优势<sup>[35]</sup>。

相关病原鉴定结果虽然同样显示链格孢属(*Alternaria* sp.)、葡萄孢属(*Botrytis* sp.)、青霉属(*Penicillium* sp.)、根霉属(*Rhizopus* sp.)和毛霉属(*Mucor* sp.)是樱桃采后主要病原,但在种水平上同其他研究存在一定差异,如:赵倩<sup>[33]</sup>发现草酸青霉(*Penicillium oxalicum*)为山西红玛瑙樱桃采后真菌病原;赵远征等<sup>[36]</sup>发现链格孢(*Alternaria alternata*)为大连大樱桃采后病原真菌;何煜波等<sup>[50]</sup>发现匍枝根霉(*Rhizopus stolonifer*)为樱桃采后病原真菌;张娜等<sup>[51]</sup>发现葡萄孢菌(*Botrytis cinerea* Pers. ex Fr.)为大连雷尼与拉宾斯、河北红灯和山东拉宾斯樱桃的采后病原真菌;杜小琴<sup>[5]</sup>发现毛霉属(*Mucor* sp.)为拉宾斯

樱桃采后病原真菌。说明不同品种、不同产地的病原菌种类存在一定种间差异。另外,虽然目前也已有研究发现 *R. oryzae*、*F. dlamini*<sub>ii</sub>、*F. fujikuroi* 和 *M. hiemalis* f. *hiemalis* 分别是龙牙百合软腐病<sup>[40]</sup>、桑树根腐病<sup>[41]</sup>、墨兰与蝴蝶兰茎基腐病<sup>[45]</sup>和鱼类<sup>[48]</sup>病原真菌,但相应种首次在红灯樱桃中被鉴定出来,说明近年来红灯樱桃中出现了一些新病害,相关研究结果对樱桃产地采前、采后病害管理有重要预警意义。

另外,由于目前多数研究表明植物病害发生多为复合病原相互作用的结果<sup>[39, 52]</sup>,因此在明确红灯樱桃采后主要病原真菌后,笔者还从广谱抑菌角度对前期发现的拮抗细菌 *B. velezensis* G-1 的脂肽物质和挥发性物质 2,4-二叔丁基苯酚的樱桃采后病害广谱抑菌效果进行了探究。研究结果虽然与 Gao 等<sup>[31]</sup>(2,4-二叔丁基苯酚对番茄 *Alternaria solani* 和 *Botrytis cinerea* 具有较好抑菌效果)、Zhang 等<sup>[39]</sup>(2,4-二叔丁基苯酚对树莓 *Botrytis cinerea* 具有较好防治效果)、Saoussen 等<sup>[53]</sup>(脂肽物质对马铃薯 *F. oxysporum*, *F. solani*, *F. graminearum* 和 *F. sambucinum* 等具有较好抑菌效果)和 Zhang 等<sup>[54]</sup>(脂肽物质对小麦 *Gaeumannomyces graminis* var. *tritici* 具有较好抑菌效果)相似,但本研究结果不仅首次发现脂肽物质对 *M. hiemalis* f. *hiemalis*、*R. oryzae*、*F. dlamini*<sub>ii</sub> 具有抑菌作用,而且首次发现挥发性物质 2,4-二叔丁基苯酚对 *M. hiemalis* f. *hiemalis*、*F. dlamini*<sub>ii</sub> 具有良好的抑菌效果。另外,虽然 2,4-二叔丁基苯酚无法有效抑制 *P. crustosum* 菌丝体生长,但可有效抑制其孢子产生,说明 2 种物质在樱桃采后真菌病害广谱防治方面具有良好的应用潜力。

最后,虽然在本研究中已对红灯樱桃采后病原真菌种类进行了较为系统鉴定,但使用 ITS 序列分析和形态鉴定仅可将病原 YT-5 和 YT-7 鉴定为链格孢属,后期还需采用分子标记、MOLDI-TOF-MS/MS、DNA 分子杂交等技术进一步确定 YT-5 和 YT-7 的分类地位。另外,虽然发现 *B. velezensis* G-1 脂肽物质和挥发性物质 2,4-二叔丁基苯酚对红灯樱桃采后病害在平板上具有较好的广谱抑菌效果,但有关 2 种抑菌物质的生物安全性、使用剂量、制剂化、贮藏应用效果和应用技术开发等问题在后期还需进一步探究,以期为樱桃采后病害生物防治提供新思路。

## 4 结 论

红灯樱桃采后病原真菌主要有米根霉 YT-1(*R. oryzae*)、镰刀菌 YT-2(*F. dlamini*<sub>ii</sub>)、藤仓镰刀菌 YT-4(*F. fujikuroi*)、链格孢属 YT-5 和 YT-7(*Alternaria* sp.)、天竺葵葡萄孢 YT-6(*B. pelargonii*)、冻土毛霉 YT-8(*M. hiemalis* f. *hiemalis*)和 YT-9 皮壳青霉(*P. crustosum*),其中镰刀菌属病原真菌(*F. dlamini*<sub>ii</sub> 和 *F. fujikuroi*)首次在红灯樱桃果实中被分离鉴定出来,对后期相应病害田间防治和采后镰刀菌毒素污染监测具有较好的预警作用。*B. velezensis* G-1 脂肽对除 YT-8(*M. hiemalis* f. *hiemalis*)和 YT-9(*P. crustosum*)外的其他病原抑菌率可超过 70%;挥发性抗菌物质 2,4-二叔丁基苯酚对除 YT-9(*P. crustosum*)外的其他病原抑菌率可超过 95%。另外,2 种物质虽对 YT-9(*P. crustosum*)抑菌效果较差,但仍可有效抑制该菌株产孢,说明 2 种抗菌物质对樱桃采后病原真菌具有良好的抗菌谱,应用潜力良好。

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