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梨树腐烂病拮抗真菌JK2的分离和鉴定

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摘 要:【目的】分离鉴定对梨树腐烂病具有拮抗活性的内生真菌,为梨树腐烂病生物防治提供新的资源菌。**【方法】**利用组织分离法从梨树腐烂病发病枝条中分离内生菌,通过平板对峙实验筛选对梨树腐烂病菌具有拮抗作用的菌株。结合形态学和分子生物学手段,分析和确定拮抗菌株的种属。利用离体梨树枝条鉴定拮抗内生真菌对腐烂病的防治效果。**【结果】**从梨树腐烂病发病枝条中分离获得一株对梨树腐烂病菌具有强烈拮抗活性的菌株JK2,抑制率超过95%。JK2上清液对梨树腐烂病菌同样具有抑制活性。经鉴定,菌株JK2为青霉属产紫青霉。离体梨树枝条鉴定结果显示,JK2对梨树腐烂病具有良好的防治效果。**【结论】**从梨树腐烂病发病枝条中分离获得一株具有拮抗活性的内生真菌JK2,该菌株是一株新的梨树腐烂病生防菌资源,具有潜在的应用价值。

关键词:梨树腐烂病;内生真菌;拮抗活性;产紫青霉

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Isolation and identification of endophytic fungus JK2 antagonistic against pear *Valsa* canker caused by *Valsa pyri*

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Abstract:【Objective】Pear is one of the most important fruits in China, where both the growing area and production of pear rank first in the world. However, the pear tree is frequently infected with certain microbial pathogens during growth. Pear *Valsa* canker disease is one of the most destructive diseases in the main production area in China, and poses a great threat to pear production. Pear *Valsa* canker disease is caused by fungus *Valsa pyri*. The pathogen can infect host bark wounded by injury, making plants exhibit reddish-brown, water-soaked, softened, and decayed symptoms. In some cases, it even can cause the whole plant death, resulting in severe production and economic losses. In recent years, pear canker disease has become more common and serious in most of planting regions of China and the development of pear industry has been greatly affected by this disease. Chemical control of this disease is still one of the most common and effective methods. However, due to the long-term use of chemical fungicides, it could increase the selection pressure on the presence of fungicides resistant isolates. In addition, it could result in environmental pollution, which greatly threatens the food safety. Therefore, it is urgently needed for new methods or means to replace chemical control of plant diseases in production. Biocontrol of plant diseases with endophytes has the characteristics of safety, low toxicity and high efficiency, which has always been the direction and hot spot of plant diseases control research. The aim of this study is to isolate and identify endophytic fungi antagonistic against pear *Valsa* canker caused by *V. pyri*, thereby providing new biocontrol fungi resources.【Methods】The pear branch infected with *V. pyri* was collected from Korla city, Xinjiang Uygur Autonomous Regions and used to isolate entophytic fungi by tissue separation method. The isolated entophytic fungi were screened by plate confrontation experiment culture

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method, to select strains antagonistic against pear *Valsa* canker caused by *V. pyri*. The supernatant of antagonistic strain was further used to test the inhibition activity. The classification of antagonistic strain was first identified by morphology method. The morphology of colony was observed by placing it on PDA medium for culture and the morphology of conidia was observed under a microscope. Subsequently, antagonistic strain was further identified by molecular biology method. Total genomic DNA of antagonistic strain was extracted with a CTAB method and used as template for PCR amplification. Two loci including rDNA-ITS (*ITS*) region and Tubulin (*Tub*) were amplified with the indicated universal primers. The PCR production was purified by using gel extraction kit. After sequencing, the obtained DNA sequences were used to blast the NCBI database. Similar sequences were used to construct phylogenetic trees using MEGA 7 software. Effect of antagonistic strain on pear canker was determined on detached branches of pear. After treatment with supernatant or conidia of JK2, the branches were inoculated with mycelia plugs of *V. pyri*.【Results】An entophytic fungus strain named JK2 was isolated from pear *Valsa* canker diseased branch. JK2 showed strong inhibitive effect on mycelia growth of *V. pyri*. Statistical result indicated that the inhibition rate of JK2 against *V. pyri* reached to above 95%. Microscopic observations revealed that JK2 resulted in abnormal mycelial morphology of *V. pyri*. After treated with JK2, the hypha of *V. pyri* became slender with less branches and part of hyphae were transparent. Statistical result showed that the treated hyphal diameter was about 3.43 μm, which was significantly reduced, comparing to CK. Different concentrations of JK2 supernatant (10%, 20%) also exhibited inhibition on mycelia growth of *V. pyri*. The inhibition rate of 10% and 20 % supernatant against *V. pyri* was 52.15% and 64.04%, respectively. This result indicated that supernatant of JK2 had the antifungal substances. Based on the result of colony and conidia morphology observation, strain JK2 was identified as *Penicillium* species. BLAST result showed that ITS sequence of JK2 was 100% similar to *Penicillium funiculosum*, *Penicillium oxalicum* and *Penicillium purpurogenum*, which could not identify the specific species of JK2. We further used *Tubulin* gene sequences to construct the phylogenetic tree, which indicated that JK2 was most closely related to *Penicillium purpurogenum* and the similarity rate was above 99.5%. Therefore, strain JK2 was identified as *Penicillium purpurogenum*. The result of pathogenicity test showed that both supernatant and conidia of JK2 could inhibit pear *Valsa* canker disease caused by *V. pyri*. Comparing to CK, supernatant treatment did not affect the disease incidence, and however the lesion length was greatly inhibited. Conidia suspension could both significantly reduce disease incidence and lesion length. These results indicated that strain JK2 was an effective endophytic fungus to control the pear *Valsa* canker disease.【Conclusion】An endophytic fungus, *Penicillium purpurogenum* JK2, was isolated form diseased pear branch. JK2 showed strong antagonistic activity against pear *Valsa* canker caused by *V. pyri*, which indicated that strain JK2 was a potential biocontrol fungus resource of pear *Valsa* canker.

Key words: Pear *Valsa* canker; Endophytic fungus; Antagonistic activity; *Penicillium purpurogenum*

梨是我国最重要的水果之一，在各地区均有栽培。但近年来，我国梨在生产过程中受到多种病害的威胁，其中腐烂病是为害最严重的病害之一[1-3]。梨树腐烂病是由病原菌 *Valsa pyri* 引起的一种真菌病害[4-5]，该病在我国梨主产区发生较为普遍，发病植株通常表现为树皮皮层腐烂，发病严重时引起梨树主干皮层坏死，甚至整株枯死[5-6]。目前，化学防治仍是防治果树腐烂病最有效的手段之一。但是长期使用化学农药，不仅增加环境对病原菌的选择压力，还会造成环境污染以及食品安全问题。因此，在生产上亟需寻求替代化学防治病害的新方法或新手段。其中，生物防治手段具有安全、低毒、高效等特点，是当前病害防治研究的方向和热点。

利用植物内生菌及其代谢产物防治病害是生物防治的重要举措之一，在生产中已经表现出很好的应用前景。目前，关于梨树腐烂病生物防治有少量

研究报道。例如，HASF（heat stable antifungal factor）是从产酶溶杆菌（*Lysobacter enzymogenes*）中分离鉴定的一种小分子物质，能导致梨树腐烂病菌菌丝体扭曲、顶端肿胀等畸形现象，从而抑制腐烂病菌的生长[7]。最近有研究者发现，从枯草芽孢杆菌（*Bacillus subtilis*）中提取的化合物吡啶二羧酸（dipicolinic acid）具有强烈的抑菌活性，对包括梨树腐烂病菌在内的多种病原真菌均表现出很好的拮抗作用。进一步实验表明，吡啶二羧酸通过抑制病原菌几丁质的合成致使细胞死亡，从而拮抗病原菌的生长。吡啶二羧酸具有很好的稳定性，并能从树皮表面渗透到韧皮部，抑制腐烂病菌侵染寄主[8]。但总体而言，当前梨树腐烂病防治的研究大部分集中在化学药剂的筛选和利用方面[9-13]，生物防治有关的研究还很少[14]，可利用的梨树腐烂病菌拮抗菌资源还很有限，仍需进一步挖掘和研究。

在本研究中，笔者从梨树腐烂病发病枝条中分离内生真菌，筛选对腐烂病菌具有良好拮抗活性的菌株，并分析拮抗菌株对腐烂病的防治效果，为腐烂病的生物防治提供资源菌。

1 材料和方法

1.1 材料

梨树腐烂病菌（*Valsa pyri*）菌株lfl-XJ是从库尔勒香梨感病枝干上分离获得，保存于中国农业科学院郑州果树研究所。

1.2 方法

1.2.1 内生真菌的分离和鉴定 参照王丽等[15]描述的组织分离法分离梨树枝干韧皮部内生真菌。经单孢纯化后得到单一菌株，并保存备用。

1.2.2 内生真菌拮抗活性筛选 采用平板对峙实验[16]，测定内生真菌对梨树腐烂病菌生长的抑制效果。用直径为0.5 cm的打孔器从内生真菌菌落边缘打取菌饼，置于PDA平板上，在距中央位置2 cm的四周各放置一块菌饼。培养2 d后，在平板中央接种直径为0.5 cm的梨树腐烂病菌菌饼，每次共5个平皿，3次重复。对照组不放置内生真菌。于25℃下黑暗培养6 d后，采用十字交叉法测量梨树腐烂病菌菌落直径，计算抑制率。抑制率/%=（对照菌落直径−处理菌落直径）/（对照菌落直径−0.5）×100。利用超景深三维立体显微镜观察梨树腐烂病菌菌丝形态特征，并测量菌丝直径。

1.2.3 拮抗内生真菌的鉴定 培养特征观察：观察内生真菌在培养基上菌落形状、颜色等，并利用显微镜观察菌丝、分生孢子的形态等特征，初步确定内生真菌种属[17]。

分子生物学鉴定：利用CTAB的方法提取内生真菌基因组DNA[18]，利用ITS和微管蛋白*Tubulin*基因通用引物分别扩增[19-20]。PCR扩增产物，经1.2%（*w*）的琼脂糖凝胶电泳检测后，切胶回收，送至北京六合华大基因科技有限公司测序。将测序结果在NCBI数据库中进行BLAST比对分析，得到与其序列同源性较高的相关模式菌株的序列，采用MEGA 7软件以邻接法构建系统发育树，进一步确定拮抗菌株的种属。

1.2.4 拮抗内生真菌上清液对腐烂病的抑制作用 接种内生真菌于PDB培养基中，于25 ℃、180 r·min^{-1}培养7 d，离心后收集上清液。配制不同含量（*w*）上清液（10%和20%）的PDA平板，以不含上清液的PDA平板作为对照，在平板中央接种梨树腐烂病菌菌饼，于25 ℃下黑暗培养6 d后，测量菌落直径，计算抑制率。

1.2.5 拮抗内生真菌对腐烂病的防治作用 利用离体梨树枝条鉴定内生真菌对腐烂病的防治作用，具体方法如下：选取健康、长势一致当年生梨树枝条，经无菌水清洗干净后，用75%（*φ*）乙醇擦拭消毒，再用无菌水清洗，晾干。用直径为0.5 cm的打孔器在枝条打孔，孔深至木质部。分别用内生真菌上清液或分生孢子（10^8个•mL^{-1}）喷施处理打孔枝条，以无菌水处理作为阴性对照，戊唑醇（安徽省银山药业有限公司）处理作为阳性对照。处理后将枝条置于培养箱中保湿2 d后，每个孔接种1个梨树腐烂病菌菌饼，再用蘸无菌水的脱脂棉缠绕，外用封口膜缠绕保湿，置于25 ℃下光照培养箱中培养，7 d后测量病斑长度，并拍照。每次接种20个孔，3次重复。发病率/%=（总接种点数−无病症的接种点数）/总接种点数×100。

2 结果与分析

2.1 梨树腐烂病菌拮抗真菌JK2的分离

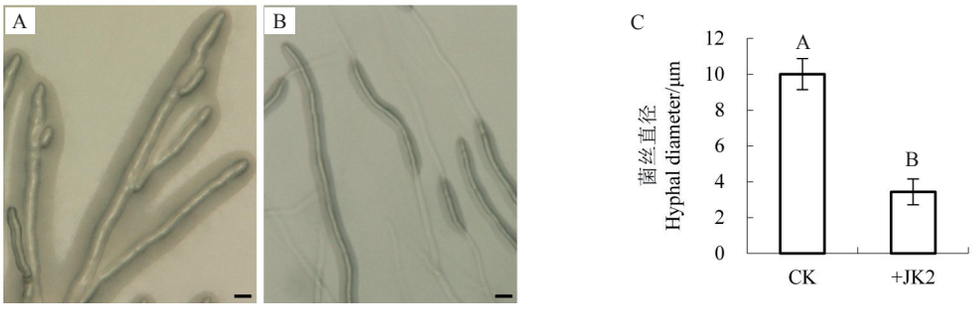
从梨树腐烂病发病枝条中分离腐烂病病原菌时，获得一株内生真菌，编号为JK2。平板对峙实验结果显示，JK2对梨树腐烂病菌具有很好的拮抗作用。当对照组的梨树腐烂病菌长满培养皿时，对峙

实验中的梨树腐烂病菌几乎完全被抑制（图1）。统计结果显示，JK2对梨树腐烂病菌的抑制率达到95.15%。

利用显微镜进一步观察菌丝形态特征，发现与对照相比，经JK2拮抗处理的梨树腐烂病菌菌丝纤细，部分菌丝呈透明状，分支变少（图2-A~B）。菌丝直径测量结果显示，对照菌丝直径约为10.01 μm，而拮抗处理后菌丝直径仅为3.43 μm（图2-C）。这些结果表明，JK2能显著影响梨树腐烂病菌菌丝形态。

图1　菌株JK2对梨树腐烂病菌的抑制作用

Fig. 1　Inhibition effect of strain JK2 on *Valsa pyri*



A. 正常梨树腐烂病菌菌丝；B. 受JK2拮抗处理后的梨树腐烂病菌丝；C. JK2对梨树腐烂病菌菌丝直径的抑制。标尺为1 cm。不同大写字母代表差异显著（Tukey's tests，$p < 0.01$）。下同。

A. Normal mycelia of *V. pyri*; B. Mycelia of *V. pyri* treated with JK2; C. Effect of JK2 on hyphal diameter of *V. pyri*. Bar = 1 cm. Different capital letters indicate significant different at $p < 0.01$ based on Tukey's tests. The same below.

图2　JK2对梨树腐烂病菌菌丝的抑制

Fig. 2　Effect of JK2 on mycelia of *V. pyri*

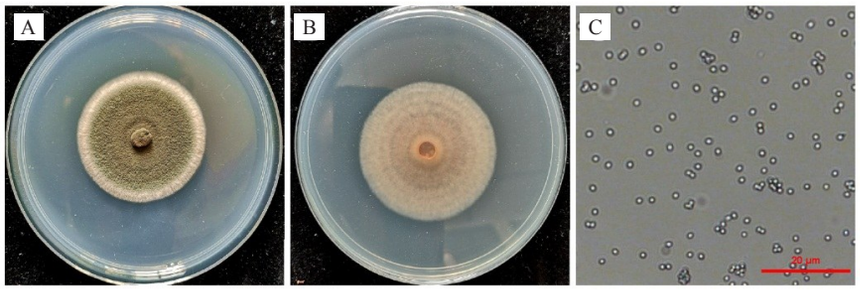
2.2　JK2上清液对梨树腐烂病菌的拮抗作用

为了进一步明确JK2对梨树腐烂病菌的抑制作用，检测了JK2上清液对梨树腐烂病菌的拮抗活性。结果显示，对照菌落平均直径为（7.23±0.09）cm时，不同含量的上清液（10%和20%）处理后的菌落平均直径分别为（3.72±0.11）cm和（2.92±0.09）cm，抑制率分别为52.15%和64.04%。这表明上清液中含有抑菌活性物质。

2.3　JK2的鉴定

JK2在PDA培养基上生长7 d后，菌落为圆形，无突起，呈灰绿色，有同心圆轮廓，背面淡黄色（图3-A~B）。菌丝致密，产孢量大，分生孢子椭圆形，表面光滑（图3-C）。初步鉴定为青霉属*Penicillium*。

采用通用引物ITS1和ITS4对菌株JK2的ITS片段进行扩增，经测序分析后，将该序列在NCBI数据库中进行BLAST比对分析，发现该菌株与绳状青霉（ITS GenBank登录号为HQ115695.1）、产紫青霉（ITS GenBank登录号为MT074698）和草酸青霉



A. JK2菌落正面；B. JK2菌落背面；C. JK2分生孢子。

A. Front view of JK2 colony; B. Back view of JK2 colony; C. Conidia of JK2.

图3　菌株JK2菌落及分生孢子形态特征

Fig. 3　Colony and conidia morphology of strain JK2

（ITS GenBank登录号为KF150220）的同源率均达到100%。为了鉴定JK2属于青霉属的哪一种，进一步利用微管蛋白基因*Tubulin*通用引物对JK2进行了扩增和测序。将测序结果与GenBank中的已知序列进行比对，并构建系统发育树。系统发育树显示，JK2与4个产紫青霉菌株位于同一个发育树分支，进化距离最近，相似率超过99.5%（图4）。因此，将菌株JK2鉴定为青霉属产紫青霉。

2.4 JK2对梨树腐烂病的防治作用

为了了解JK2对梨树腐烂病的防治效果，利用离体梨树枝条进行了鉴定。鉴定结果显示，接种梨树腐烂病菌7 d后发现，对照水处理的梨树枝条表现出明显的腐烂病病斑症状，而JK2上清液或分生孢子悬浮液处理的枝条腐烂病发病程度显著降低，阳性对照戊唑醇处理后的枝条无病斑症状（图5-A）。进一步统计结果发现，与对照处理相比，上清液处理不影响腐烂病的发病率（图5-B），但能显著抑制腐

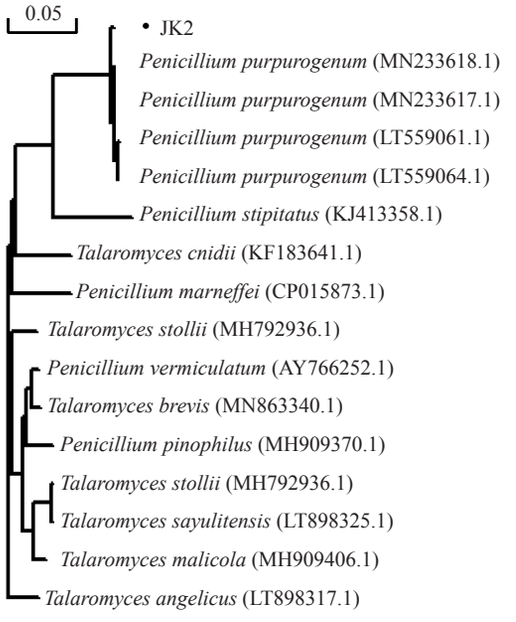
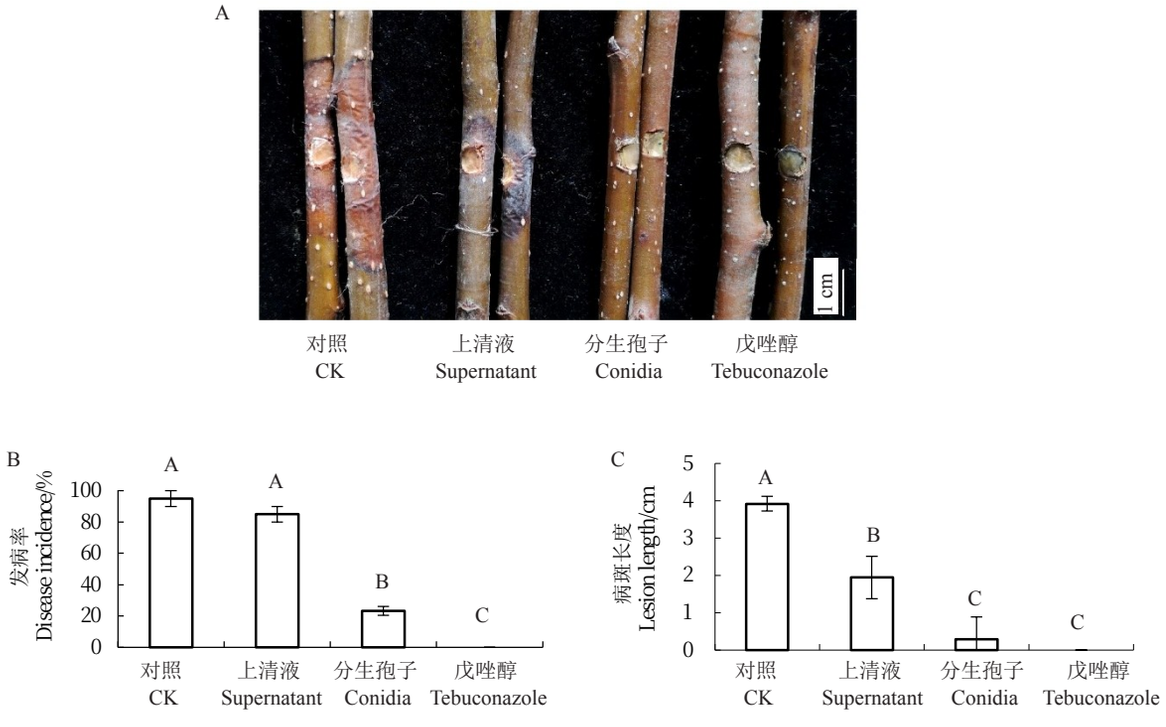


图4 基于*Tubulin*基因序列构建的系统发育树

Fig. 4 The phylogenetic tree based on sequences of *Tubulin* gene



A. JK2能拮抗梨树腐烂病的发病程度；B. 梨树腐烂病发病率统计；C. 梨树腐烂病病斑长度统计分析。用上清液或分生孢子悬浮液喷施处理梨树枝条，再接种梨树腐烂病菌。接种7 d后调查病斑长度。无菌水处理作为对照，戊唑醇处理作为阳性对照。

A. JK2 could antagonize pear canker; B. Statistical analysis of the disease incidence; C. Statistical analysis of lesion length. After treatment with supernatant or conidia of JK2, the pear branches were inoculated with *Valsa pyri*. The lesion length was measured at 7 days post inoculation. Water treatment was used as negative control and tebuconazole treatment was used as positive control.

图5 JK2对梨树腐烂病的发病程度的影响

Fig. 5 Effect of JK2 inhibition on pear *Valsa* canker

烂病病斑扩增（图5-C）。分生孢子悬浮液处理枝条发病率约23.33%，平均病斑长度为0.29 cm，均显著低于对照（图5-B~C）。以上结果表明，内生真菌JK2对梨树腐烂病具有较好的防治潜力。

3　讨　论

梨树腐烂病是为害梨生产最严重的真菌病害之一，但目前还没有对腐烂病防治效果显著的化学药剂，常常造成化学农药乱用多用的问题，严重危害环境。利用内生菌防治植物病害是生物防治重要的措施之一，然而当前可利用的梨树腐烂病生防菌资源还很有限。最近，研究人员从果蔬酵素液和香梨枝条中分离内生菌，筛选获得12株具有梨树腐烂病菌拮抗活性细菌，其中腐烂病防治效果最好的2个菌株均为贝莱斯芽孢杆菌 *Bacillus velezensis*[14]。笔者在本研究中从梨树腐烂病发病枝条中分离获得一株真菌JK2，对梨树腐烂病菌具有强烈的抑制活性，抑制率超过95%。将JK2接种梨树枝条或叶片，不会导致病害症状出现，表明JK2不是致病菌株，只是一株内生真菌。结合形态学和分子生物学鉴定结果，显示菌株JK2为青霉属产紫青霉。青霉属真菌广泛存在于自然界中，具有易培育、产孢能力强、繁殖迅速等特点，非常有利于生防制剂的制备。目前，很多研究者从不同植物或土壤中分离获得了青霉属内生真菌，并鉴定了它们对植物病害的防治效果。例如，申光辉等[21]从土壤中分离获得的灰黄青霉（*Penicillium griseofulvum*）菌株，对草莓根腐病具有较好的防治效果。后续研究发现，该灰黄青霉菌株对马铃薯土传病害病原真菌立枯丝核菌（*Rhizoctonia solani*）、茄病镰刀菌（*Fusarium solani*）、硫色镰刀菌（*Fusarium sulphureum*）和大丽轮枝菌（*Verticillium dahliae*）也具有很好的拮抗活性[22]。土壤中分离的3株绳状青霉（*Penicillium funiculosum*）菌株对柑橘疫病表现出不同程度的拮抗活性[23]。草酸青霉（*Penicillium oxalicum*）对番茄枯萎病具有较好的生防效果[24]。最新研究发现，产紫青霉对烟草黑胫病菌（*Phytophthora parasitica* var. *nicotianae*）和根黑腐病菌（*Thielaviopsis basicola*）均有较强的拮抗作用[25]。目前尚未见利用青霉防治果树腐烂病的研究报道。因此，本研究中分离鉴定的具有拮抗活性的产紫青霉菌株JK2是一种新的梨树腐烂病生防菌资源。

筛选鉴定内生菌中抑菌活性物质被认为是寻找新的活性化合物的有效途径，也是开发新型生物农药的重要手段[26-27]。已有研究显示，利用从产酶溶杆菌分离鉴定的多肽HASF制备成的凝胶剂，对梨树腐烂病具有很好的防治效果，并且该制剂可与化学农药优势互补，能有效降低化学农药的使用量，有望开发成生物源农药[7]。本研究表明，JK2上清液对梨树腐烂病菌同样具有较好的拮抗活性，能显著抑制梨树腐烂病菌菌落生长，说明上清液中含有抑菌活性物质。目前，笔者正在对JK2上清液中的化合物进行鉴定，以期筛选获得具有抑菌活性的物质。

离体枝条鉴定试验显示，菌株JK2对梨树腐烂病具有较好的防治效果，其中JK2分生孢子悬浮液处理对腐烂病的防治效果与阳性对照戊唑醇处理相当，表现出潜在的应用价值。后续笔者将对JK2在田间防治梨树腐烂病的效果及其安全性进行评估和研究，为JK2在梨树腐烂病防治中的广泛应用奠定基础。

4　结　论

从梨树腐烂病菌发病枝条中分离获得一株内生真菌JK2，鉴定为青霉属产紫青霉。JK2对梨树腐烂病菌具有强烈的抑制活性，并对梨树腐烂病表现出较好的防治效果，是一种新的梨树腐烂病生防菌资源。

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