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苹果根腐病根际土壤真菌组成及多样性研究

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摘 要:【目的】探究苹果感染根腐病后根际土壤真菌群落组成、丰富度及多样性变化。**【方法】**以ITS5-1737F和ITS2-2043R为引物,对真菌ITS区进行扩增并利用Illumina Hiseq高通量测序技术进行测序,分析苹果根腐病病株及健康植株根际土壤真菌物种组成、丰度及α-多样性。**【结果】**通过高通量测序,患根腐病和健康根际土壤分别获得1 006、708个OTU,主要分为11个门,33个纲,148个科,263个属。病、健根际土壤真菌种群多样性、丰富度均存在显著性差异。患病土样中,子囊菌门相对丰度显著低于健康土样,Dothideomycetes纲相对丰度极显著低于健康土样,而Leotiomycetes、Eurotiomycetes纲相对丰度显著低于健康土样;已报道的与苹果根腐病相关的病原菌*Fusarium*、*Rosellinia*相对丰度高于健康土壤,但差异不显著;有益真菌*Trichoderma*相对丰度稍低于健康土样。**【结论】**苹果感染根腐病后,根际土壤的真菌物种多样性、群落多样性升高,物种均匀度下降。根腐病病原菌和有益真菌普遍分布在患病和健康根际土壤中。患病土样中,已报道的苹果根腐病病原菌高于健康土壤,而部分有益菌,如*Trichoderma*低于健康土壤,说明病原菌的略微积累和有益菌的略微下降,都可能打破根际土壤真菌群落的平衡,影响土壤的健康。

关键词:苹果;根腐病;根际土壤;真菌群落;高通量测序;α-多样性

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Fungal community and diversity in rhizospheric soil with root rot in an apple orchard

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Abstract:【Objective】The relative abundance, community composition and diversity of fungi were investigated to reveal the effects of root rot disease on rhizospheric soil microbe. Meanwhile, the reported pathogens associated with root rot disease were examined and the potential biocontrol agents were predicted.【Methods】Rhizospheric soil from healthy and root rot trees in the same orchard were collected, which were denoted as Healthy and Diseased. By digging up the top soil, the roots were exposed. A part of the roots was cut and shaked gently to remove the loosely adhering soil, and then the tightly adhering soil was collected. Then, the soil samples were put into liquid nitrogen and stored in an ultralow temperature freeze at −80 ℃ for DNA extraction. The total DNA was extracted from each sample using the Fast DNA Spin Kit for soil. With ITS5-1737F and ITS2-2043R as the primer pair, the fungal internal transcribed spacer (ITS) region of rDNA was amplified and then sequenced by means of Illumina HiSeq high-throughput sequencing technology. The high quality paired-end reads were merged by FLASH. The operational taxonomic units (OTUs) were obtained with 97% similarity cutoff using

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UPARSE. Based on the rarefying OTU relative abundance for taxa, the taxonomy of each ITS sequence was analyzed by RDP Classifier algorithm against the UNITE 7.2 using a confidence threshold of 70%. The significant difference in alpha-diversity were calculated using student's T test with Mothur v.1.30. 【Results】Approximately 0.58 million high quality sequence tags were obtained. The rarefaction curves showed that as the numbers of reads increased, the sobs index tended to be gentle, so the sequenced results were reasonable and could reflect the actual situation of collected samples. Finally, Healthy and diseased rhizospheric soil samples got 708 and 1 006 OTUs, respectively, mainly affiliated to 11 phylum, 33 classes, 148 families, and 263 genera. The result showed that 611 OTUs were detected both in healthy and diseased samples, while 97 OTUs and 395 OTUs were respectively observed only in healthy and diseased soil. The alpha-diversity index (Sobs, Shannon, Simpson, ACE, Chao) analysis showed that Sobs, Shannon, ACE, and Chao index in diseased soil were significantly higher than in healthy soil, while Simpson index was lower than that with healthy soil. Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Mortierellomycota and Rozellomycota were observed in all samples. The dominant phylum included Ascomycota, Basidiomycota and Chytridiomycota. Especially Ascomycota possessed absolute advantage. In addition, there was a large amount of unclassified fungi in all samples. Ascomycetes fungi in diseased soil were significantly lower (p=0.045) than those in healthy soil. At class level, communities with relative abundance above 1% were arranged as Dothideomycetes, Sordariomycetesa, Leotiomycetes, Tremellomycetes, Eurotiomycetes and Mortierellomycetes. The relative abundance of Dothideomycetes in healthy soil was significantly higher than that in diseased soil. Moreover, Leotiomycetes and Eurotiomycetes were lower than those in diseased soil. At genus level, healthy soil was enriched with more unclassified *Pleosporales*, *Plectosphaerella* and *Cladosporium*, and unclassified *Nectriaceae*, *Ilionectria*, *Guehomyces*, *Trichoderma*, *Phoma* and *Lophiostoma*. While diseased soil was enriched with more unclassified Ascomycota, *Pseudogymnoascus*, *Fusarium*, *Naganishia*, *Mortierella*, *Trimmatostroma* and *Knufia*. In order to analyze the potential pathogens that caused apple root rot disease, the relatively abundance of reported pathogens associated with fruit root rot were calculated. The result showed that seven genera(*Alternaria*, *Cylindrocarpon*, *Cylindrocladiella*, *Fusarium*, *Ilyonectria*, *Pestalotiopsis* and *Rosellinia*)were observed both in healthy and diseased soil. Among those genera, *Fusarium* and *Rosellinia* that had been reported causing apple root rot were enriched in the root rot soil, but the difference between health and disease soil was not significant. At the same time, the potential biocontrol agents such as *Trichoderma*, *Penicillium* and *Paecilomyces* existed in both healthy and diseased soil. *Trichoderma* was enriched in healthy soil, and *Penicillium* was enriched in diseased soil. 【Conclusion】The OTUs numbers, observed species, fungal diversity in the rhizospheric soil of root rot were significantly higher than those in healthy soil. Infected by the root rot disease, more fungi would appear in rhizospheric soil. The dominant fungal communities at phylum level were Ascomycota, Basidiomycota and Mortierellomycota. Dominant classes included Dothideomycetes, Sordariomycetes, Leotiomycetes, Tremellomycetes, Eurotiomycetes and Mortierellomycetes. The pathogens *Fusarium* and *Rosellinia* associated with apple root rot had higher relative abundance in the diseased soil than in the healthy soil, so we speculated that *Fusarium* and *Rosellinia* maybe were the mainly pathogens causing apple root rot in this orchard. However, more laboratory separation experiment and pathogenicity detection should be undertaken to validate the speculation. Meanwhile, potential biocontrol agent *Trichoderma*, *Penicillium* and *Paecilomyces* were observed both in healthy and diseased soil. Although we could not clearly judge which agent played a crucial role by high-throughput sequencing, some beneficial clues can be gotten to rapidly find out possible biocontrol fungi.

Key words: Apple; Root rot; Rhizosphere soil; Fungal community; High-throughput sequencing; Alpha-diversity

苹果是重要的落叶果树，是世界上栽培面积最广、产量最多的果树之一[1]。我国是世界上最大的苹果生产国和消费国。据统计，2017 年，我国苹果总产量 4 450 万 t，占世界苹果产量的 58%，苹果消费 3 838 万 t，占世界苹果总消费的 59%[2]。随着产业的发展，苹果根部病害逐年加重，严重威胁苹果树体的健康。其中由土传病原真菌引起的根腐病是重要的根部病害之一。根腐病具有类型多样、病原菌复杂的特点，在世界苹果生产国均有报道，被认为是目前最严重的苹果根部土传病害[3]，严重影响产业的健康稳定发展。

植物通过根际吸收根际土壤中的营养，并通过根际沉积对根际土壤产生影响[4]。在自然条件下，根际-土壤之间的相互反馈作用是一个复杂的过程，被认为是植物群落动态与营养循环的主要驱动力[5]，而根际微生物在这一反馈系统中发挥着重要的作用。根际微生物既可以通过养分竞争、拮抗作用和诱导系统抗性等机制抑制土壤中病原菌，进而促进植物生长[6]，也可以通过病原菌的积累而导致植株的大量死亡[7]。因而，研究苹果感染根腐病后，根际土壤真菌群落组成及多样性变化，对深入了解苹果根腐病发生的原因及采取相应的防治策略具有重要意义。基于 Illumina 测序的宏基因分析可以高效、快速、准确检测微生物群落的多样性，为揭示土壤微生物丰度、物种组成提供大量的信息，该项技术已被广泛运用于根际土壤微生物的研究。本文通过高通量测序结果，研究苹果感染根腐病后根际土壤真菌的多样性变化，为利用根际微生物防治根腐病提供参考。

1　材料和方法

1.1　土壤样品

患根腐病和健康根际土样于 2018 年 9 月采集自云南省昭通市昭阳区洒渔乡苹果种植园（北纬 27°28′39″，东经 103°36′35″），海拔 2 050 m。果园面积 0.53 hm^2，树龄 15 a，砧木为昭通海棠，品种为‘红富士’；当地年平均气温 11 ℃，年降雨量 729.7 mm。该果园于 2018 年春季发现部分植株新生枝条较少，叶片变小，夏季整株生长较弱。9 月，在果园中选择整株叶片变黄、新生枝条较少的病树，距离主干 30 cm 处挖至 25~30 cm 深，取出表面颜色变棕色、褐色的根系将其表面黏附的土壤抖落在事先灭菌的锡箔纸上，去除植物残体包好，标记为 Disease 1 放到液氮中；与病株相隔一株，选取一株叶片健康、新生枝条丰富、长势健壮且 4 棵相邻植株均无发病的植株，以同样的方式采集健康根际土壤，标记为 Health 1。在距离病株 5 株以上的位置，寻找另一症状相似的病株，采集患病根际土壤，相隔一株的健康植株采集健康土样，同样的方法采集根腐病根际土壤共 6 份，标记为 Disease 1~ Disease 6，健康根际土壤 6 份，标记为 Health 1~ Health 6。

样品运回实验室后，从液氮中取出迅速转移到−80 ℃冰箱保存，干冰保存送至测序公司提取 DNA，进行高通量测序。

1.2　测序与数据处理

1.2.1　*土壤微生物基因组总 DNA 提取及 ITS 序列的扩增*　土壤总 DNA 的提取使用美国 MP 公司 Fast DNA™ SPIN Kit for Soil 试剂盒。提取 DNA 后利用琼脂糖电泳检测 DNA 的纯度和浓度，用灭菌的超纯水稀释至 1 ng·μL^{-1} 保存于−80 ℃以备用。

以 ITS5-1737F（GGAAGTAAAAGTCGTAACAAGG）和 ITS2-2043R（GCTGCGTTCTTCATCGATGC）为引物对 ITS 区真菌进行扩增，扩增体系为 20 μL（4 μL 5 × Fastpfu Buffer，2 μL 2.5 mmol·L^{-1} dNTP，0.8 μL 引物 1737F，0.8 μL 引物 2043R，0.4 μL Fastpfu Polymerase，0.2 μL BSA，10 ng DNA）。扩增后取 3 μL 利用 2%琼脂糖凝胶电泳检测 PCR 产物。

1.2.2　*数据分析*　利用 FLASH 软件对原始数据进行拼接，将拼接得到的序列进行质量过滤后得到高质量的 Tags 序列。真菌 Alpha 多样性采用 Mothue version v.1.30。稀释曲线、Veen 图、Spearman、热图等分析结果均由 R 软件分析和生成。

2　结果与分析

2.1　患根腐病、健康苹果植株根际土壤真菌的 OTU 分类

患根腐病、健康根际土壤中分别检测到 1 006、708 个 OTU（图 1），其中共有 611 个 OTU，健康土样特有 97 个 OTU，患根腐病土样特有 395 个 OTU，说明感染根腐病后，根际土壤真菌 OTU 数量增加。

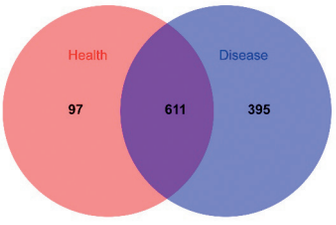


图1 健康和患根腐病根际土壤OUT数量关系

Fig. 1 Numerical relationship of the OTUS between health and root rot apple rhizosphere soil

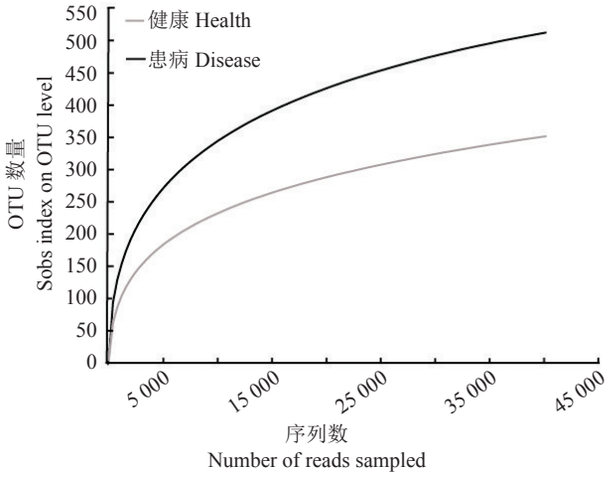


图2 稀释曲线

Fig. 2 Rarefaction curves

2.2 患根腐病、健康苹果植株根际土壤真菌α-多样性分析

稀释曲线可以反映测序数据量的合理性，如图2所示，健康和患根腐病土样稀释曲线随着测序量的增大，所观察到的物种数量趋于平缓，达到饱和，且每组样本DNA文库的覆盖率都在99%以上，说明本次测序的数据量合理，能较好地反映样品的真实情况。

对健康和患根腐病根际土壤真菌进行α-多样性分析（表1），结果表明患根腐病土样真菌OTU数量、物种种类、种群多样性指数Shannon指数以及种群丰富度指数Chao指数、ACE指数均显著高于健康根际土壤，而物种均匀度指数Simpson指数显著低于健康根际土壤。

表1 健康和患根腐病根际土壤真菌α-多样性

Table 1 Fungal alpha-diversity of health and root rot apple rhizosphere soil

样品 Sample	物种种类 Species	OTU数量 OTUs	Sobs指数 Sobs index	Shannon指数 Shannon index	Simpson指数 Simpson index	ACE	Chao指数 Chao index
患病 Disease	357 a	1006 a	512.00a	4.0053 a	0.047 b	631.53 a	640.59 a
健康 Health	302 b	708 b	351.17b	3.0492 b	0.133 a	467.91 b	472.82 b

注：同列数据后不同小写字母表示差异显著（$p<0.05$）。下同。

Note: Different small letters in the same column indicated significant difference at 0.05 level. The same below.

2.3 患根腐病、健康苹果植株根际土壤真菌不同分类水平上的丰度分析

对健康和患病根际土壤真菌在不同分类水平进行群落组成分析。门分类水平分析表明，健康和患病土样均观察到子囊菌门（Ascomycota）、担子菌门（Basidiomycota）、壶菌门（Chytridiomycota）、球囊菌门（Glomeromycota）、Mortierellomycota、Mucoromycota、Rozellomycota和未知真菌（图3）。其中，壶菌门、球囊菌门、Mucoromycota、Rozellomycota四个门的相对丰度均在1%以下，合并为Others。对两组样本的优势菌群进行差异性分析，结果表明，健康土样中子囊菌门的相对丰度显著高于（$p=0.045$）患病土样，其他门无显著性差异。纲分类水平分析表明，相对丰度大于1%的菌群，按照在土样中的占比从大到小依次是座囊菌纲（Dothideomycetes）、粪壳菌纲（Sordariomycetes）、锤舌菌纲（Leotiomycetes）、银耳纲（Tremellomycetes）、散囊菌纲（Eurotiomycetes）、被孢霉纲（Mortierellomycetes）。其中，健康土样中的座囊菌纲相对丰度极显著高于病

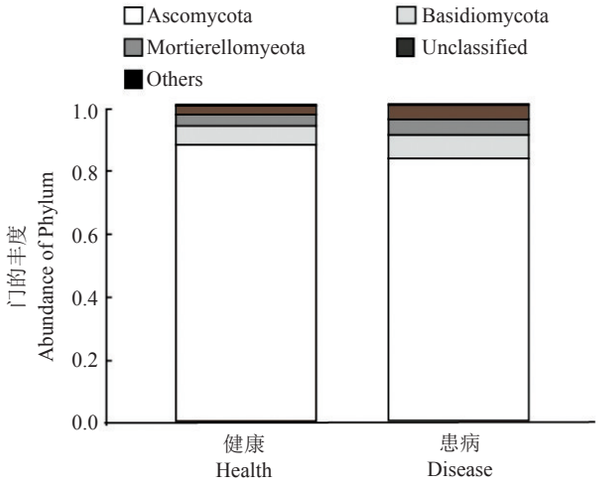
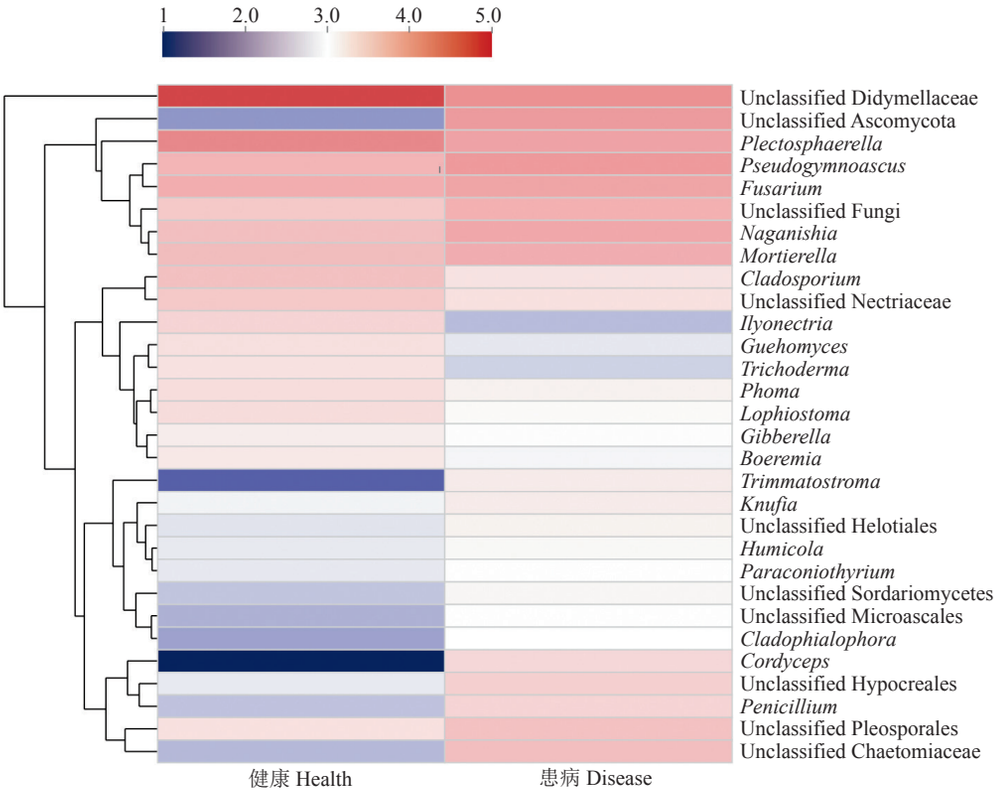


图3 健康和根腐病根际土壤真菌门组成柱形图

Fig. 3 Composition barplot of health and root rot apple rhizosphere soil on phylum level

株土样（$p < 0.01$），而锤舌菌纲和散囊菌纲的相对丰度则显著低于病株土样，其余菌群无显著性差异。

为更直接地显示不同样本中真菌类群的相似性和差异，根据所有样品在属水平的物种注释及丰度信息，选取丰度排名前 30 的属，根据其在健康及患病土样中的丰度信息，绘制成热图（图 4）。结果显示，健康土样中富集较多的未知格孢腔菌目的 *Pleosporales*、*Plectosphaerella*、*Cladosporium*、未知 *Nectriaceae*、*Ilionectria*、*Guehomyces*、*Trichoderma*、*Phoma*、*Lophiostoma*，而患病根际土壤富集更多的未知子囊菌、*Pseudogymnoascus*、*Fusarium*、*Naganishia*、*Mortierella*、*Trimmatostroma*、*Knufia* 等真菌。



颜色越蓝表明丰度越低，颜色越红代表丰度越高。

The dark blue means the low abundance, the dark red means the high abundance.

图 4　土壤真菌丰度前 30 的菌属相对丰度 Heatmap 图

Fig. 4　Relative abundance heatmap of first 30 most abundant genera

为分析与苹果根腐病相关的病原真菌，根据健康和患病两组土样在属水平上的物种注释及丰度信息，从所有的分类信息中提取已报道的与根腐病相关的病原真菌进行组间差异性分析（表 2）。

表 2　已报道与果树根腐病相关病原菌的相对丰度

Table 2　Relative abundance of reported pathogenic fungus associate with fruit root rot

%

样品 Sample	相对丰度 Relative abundance						
	Alternaria	*Cylindrocarpon*	*Cylindrocladiella*	*Fusarium*	*Ilyonectria*	*Pestalotiopsis*	*Rosellinia*
患病 Disease	0.078 a	0.002 a	0.001 a	4.529 a	0.477 b	0.000 a	0.932 a
健康 Health	0.117 a	0.137 a	0.022 a	3.275 a	2.200 a	0.001 a	0.023 a

在所有的样本中，观察到已报道的与果树根腐病相关的病原菌包括 *Alternaria*、*Cylindrocarpon*、*Cylindrocladiella*、*Fusarium*、*Ilyonectria*、*Pestalotiopsis*、*Rosellinia* 七个属，其中已报道的与苹果根腐病相关的病原菌包括 *Fusarium*、*Rosellinia* 两个属。结果显示，患病根际土壤中 *Fusarium*、*Rosellinia* 的相对丰度均高于健康根际土壤，但差异并未达到显著性水平。

3　讨　论

本研究采用 Illumina Hiseq 高通量测序技术对患根腐病和健康苹果植株根际土壤真菌组成和多

样性进行测序分析，健康和患病根际土壤分别获得412 254、34 858条有效序列用于后续分析。用于后续分析的有效序列数目之多，以及稀释曲线随着测序量的增大，所观察到的物种数量趋于平缓，说明使用该方法分析根腐病和健康根际土壤真菌组成和多样性分析具有可行性。

与前人对黄连[8]、三七[9]、柑橘[10]等的研究结果相一致，在门分类水平上，患病及健康根际土壤中的主要优势菌群均为子囊菌门、担子菌门、接合菌门，其中子囊菌门在根际土壤真菌中占绝对优势。

根际土壤微生物区系与植物根系的生长和代谢密切相关，对植物营养元素的供给和植物健康的维持发挥着重要作用[11]。对根腐病及健康根际土壤的真菌多样性研究表明，根腐病根际土壤真菌在物种种类、种群多样性及种群丰富度各方面均显著高于健康苹果根际土壤，这与于慧瑛等[12]对人参根腐病的研究结果一致。

根腐病是一种普遍发生在各种中草药植物，辣椒、油豆角、黄瓜、大蒜、洋葱等蔬菜作物和草莓、蓝莓、苹果、梨、桃、牛油果等各种果树作物上的土传病害，常常由多种病原菌复合侵染造成。目前，已报道与果树相关的根腐病病原真菌包括*Alternaria*、*Clitocybe*、*Colletotrichum*、*Cylindrocladium*、*Fusarium*、*Helicobasidium*、*Ilyonectria*、*Pestalotiopsis*、*Phellinus*、*Phytophthora*、*Phytophthora*、*Phytopythium*、*Pythium*、*Rhizoctonia*、*Rosellinia*、*Sclerotium*、*Thielavilpsis*[13-26]等多个属的真菌，每种病原菌均可感染多种园艺作物。其中，已报道的苹果根腐病病原菌包括*Sclerotium*、*Fusarium*、*Rosellinia*、*Helicobasidium*、*Armillaria*、*Phytophthora*等属的20多个种[23,27]。作物的连续种植和土地的重复利用而导致的病原菌积累是土传病害发生的原因之一[7]。研究中观察到与苹果根腐病相关的病原菌*Fusarium*和*Rosellinia*两个属的相对丰度均表现为患病土壤较健康土壤高，因此推测，*Fusarium*和*Rosellinia*可能是引起该苹果园根腐病的主要病原菌，但仍需室内分离及相应的致病性检测进一步证实。

研究根腐病根际土壤的微生物结构，同时也可为探索根腐病潜在的拮抗微生物提供线索。*Trichoderma*、*Penicillium*、*Cliocladium*、*Sporidesmium*、*Paecilomyces*等真菌被证明是土传病原菌的拮抗菌[11]。在健康和患病土样中，*Trichoderma*的相对丰度分别为1.55%、0.29%；而另一种具有生防潜能的生防菌*Penicillium*的相对丰度则表现为患病土样较健康土样高，分别为1.37%、0.39%，患病根际土壤中同时也存在较多的生防菌，这可能是土壤微生态系统对病原菌的应答，更多的病原刺激了更多*Penicillium*真菌出现。此外，在患病土样中还观察到极少量的*Paecilomyces*。然而，验证这些生防菌对苹果根腐病病原菌的生防效果，仍需要进一步研究。此外，如何充分利用这些拮抗菌，并开发能发挥田间作用的产品，是关系到苹果产业健康、可持续发展的一个重要研究方向。

4 结 论

苹果感染根腐病后，根际土壤的真菌物种多样性、群落多样性升高，物种均匀度下降。根腐病病原菌和有益真菌普遍分布在患病和健康根际土壤中。病原菌的微小变化可能也会打破原有真菌种群的平衡，从而可能导致根腐病的发生。另外，苹果根际土壤中同时也存在木霉属、青霉属、拟青霉属等具有生防潜能的真菌，但利用这些菌进行苹果根腐病防治仍需要更深入的研究。

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