

耕作方式和秸秆还田对纤维素降解细菌多样性的影响^{*}

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摘 要 免耕和秸秆还田是培肥地力的重要措施, 明确其对土壤纤维素降解细菌群落的影响对于土壤质量提升具有重要意义。选择中国科学院封丘农业生态国家试验站耕作秸秆还田(W_tS)、免耕秸秆还田($W_{nt}S$)、耕作秸秆不还田(W_t)和免耕秸秆不还田(W_{nt})4种处理小区并采集土壤样品, 用CMC-Na刚果红培养基对纤维素降解细菌计数, 采用PCR-RFLP技术研究保护性耕作和秸秆还田对纤维素降解细菌群落的影响。结果表明, 秸秆还田与免耕处理均不同程度提高了纤维素降解细菌数量。 W_tS 、 $W_{nt}S$ 、 W_t 和 W_{nt} 4个纤维素降解细菌基因文库中, OTUs数量分别为23、26、20和19个, 秸秆还田加免耕处理多样性指数最高。本文获得的纤维素降解细菌共属于11个属, 秸秆还田土壤中Streptomycetaceae、Flavobacterium、Sphingobium相对丰度明显多于秸秆不还田处理土壤; 免耕处理土壤中Pseudomonas、Phyllobacterium、Paenibacillus、Promicromonosporaceae、Sphingobacterium相对丰度明显高于耕作处理土壤。典范对应分析显示四种处理纤维素降解细菌群落结构发生了较大变化, pH、全磷、有机碳和全钾与免耕处理呈显著正相关性, 全氮、碱解氮、速效钾、速效磷和有机碳与秸秆还田处理呈显著正相关性。免耕和秸秆还田能提高土壤中纤维素降解细菌数量及多样性, 改变纤维素降解细菌群落结构。

关键词 免耕; 秸秆还田; 纤维素降解细菌; 多样性; PCR-RFLP

中图分类号 Q938.1 **文献标识码** A

纤维素是一类重要的生物高聚物, 占植物干重的30%~50%^[1], 它们随植物残体进入土壤后, 为土壤微生物生长和繁殖提供丰富的碳源。不同种属的微生物产生不同的酶系^[2], 因而不同微生物来源的酶所催化的有机底物特异性有很大差别^[3]; 此外, 由于土壤中存在的纤维素种类繁多、结构复杂和性质相异, 纤维素降解微生物的系统发育分布亦十分广泛^[4]。纤维素的微生物降解不仅能够直接引起土壤有机碳库储量和组成发生变化, 而且与土壤结构稳定性和土壤肥力质量密切相关。

土壤环境中存在多种纤维素降解菌, 国内外对纤维素降解菌的分离、纯化和鉴定进行了大量

研究, 但这些研究以土壤中的真菌为主, 这是因为真菌往往具有纤维素酶产量大和提取简单等特点^[5]。近年来, 基于细菌生长快、酶系复杂、易于表达以及耐受极端环境等优点, 纤维素降解细菌的研究工作重新得到广泛重视, 已经分离了大量的好氧和厌氧纤维素降解细菌^[6-7]。土壤中的纤维素降解与纤维素降解菌的数量、活性及群落结构密切相关, 然而细菌纤维素酶基因家族众多^[8], 难以采用通用的保守引物扩增功能基因, 土壤纤维素细菌多样性的研究报道十分有限。例如, Ulrich等^[9]从砂土和壤土中分离的537株纤维素降解细菌, 菌株之间降解能力差别很大, 且与系统发育之

^{*} 国家重点基础研究发展计划项目(2011CB100504)和国家自然科学基金项目(41371262, 41471239)资助 Supported by the National Basic Research Program of China (973 Program) (No. 2011CB100504) and the National Natural Science Foundation of China (Nos. 41371262 and 41471239)

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收稿日期: 2016-01-15; 收到修改稿日期: 2016-03-29; 优先数字出版日期(www.cnki.net): 2016-04-07

间没有关联；Saha等^[10]采用培养方法从长期施用有机肥的试验小区分离了54株纤维素降解细菌，通过16S rDNA酶切分型和测序发现纤维素降解细菌有10种T-RFLP类型；Yang等^[11]对森林土壤中纤维素降解细菌研究表明，落叶阔叶林中纤维素降解细菌数量高于常绿阔叶林，这些细菌可被分为35个RFLP类型。这些研究结果暗示，土壤中纤维素降解菌存在极高的生物多样性，它们与土壤性质、植被类型、耕作和施肥方式都可能存在某些关系，值得进行深入研究。

免耕和秸秆还田是培肥地力的重要措施，免耕减少了对土壤结构的破坏，增加了底物丰度和均匀度，提高了底物利用率和底物利用多样性^[12]。已有报道指出，不同耕作方法对真菌群落结构有显著影响，翻耕和免耕形成了不同特征真菌区系，免耕秸秆覆盖的土壤真菌多样性高于翻耕^[13]。秸秆覆盖还田提高了土壤有机质含量，促进团聚体的形成，创造了更稳定的微环境，从而有利于增加微生物活性与多样性^[14-16]。但免耕和秸秆还田能否改变微生物群落结构或者提高纤维素降解细菌物种丰富度、优势度仍存在争议。本研究采用PCR-RFLP技术分析长期免耕与秸秆还田土壤中纤维素降解细菌多样性，以期为黄淮海平原保护性耕作研究及示范推广提供理论根据。

1 材料与方法

1.1 试验地概况及样品采集

试验始于2006年，地点位于河南省境内的中国科学院封丘农业生态国家试验站（35°00'N，114°32'E），属于半干旱半湿润的温带季风气候，年平均气温13.9℃，年平均降水量615 mm。耕作制度为冬小麦-夏玉米轮作，土壤类型为典型潮土和砂壤土，设置4个处理（表1），每个处理4个重

复，每个试验小区面积14 m×6.5 m。2013年12月，于各小区采用五点取样法，采集表层0~10 cm土壤样品，带回实验室于4℃和-20℃保存，具体试验设计与土壤理化性质参见文献[17]。

1.2 菌株、培养基与试剂

大肠杆菌*E. coli* DH5 为本实验室保存，Taq酶、pMD-19T载体、限制性内切酶*Hha* I和*Afa* I购自Takara公司。所用培养基为CMC-Na，PCR引物由上海英骏生物技术有限公司合成，测序由南京金斯瑞生物科技有限公司完成。

1.3 土壤中纤维素降解细菌筛选与计数

采用平板培养方法，对不同处理土壤中纤维素降解菌进行筛选与计数。具体做法为：分别称取10 g土样加入含有90 ml无菌水三角瓶中，120 r min⁻¹震荡20 min，稀释为10⁻²~10⁻⁴浓度梯度悬液，并将其涂布于CMC-Na平板上，30℃培养4~6 d。待菌落长出后点种到CMC-Na固体平板上，刚果红染色，四周有透明圈的菌株即为纤维素降解菌。

1.4 土壤纤维素降解细菌多样性的PCR-RFLP分析

采用菌落PCR方法扩增纤维素降解细菌16S rDNA，目的片段长度1 500 bp左右，引物序列：27f（5'-AGAGTTTGATCCTGGCTCAG-3'）和1492r（5'-GGTTACCTTGTTACGACTT-3'）；扩增体系（25 μl）：PCR缓冲液（10× Buffer）2.5 μl，dNTP（25 mmol L⁻¹）2.5 μl，Mg²⁺（25 mmol L⁻¹）2.5 μl，引物27f（25 mmol L⁻¹）0.3 μl，引物1492r（25 mmol L⁻¹）0.3 μl，Taq 酶（5 U μl⁻¹）0.3 μl，10%吐温80 2.0 μl，DNA模板，ddH₂O 补足25 μl。扩增程序：94℃预变性5 min；94℃变性30 s，52℃退火30 s，72℃延伸90 s，30个循环；最后72℃延伸15 min，10℃保温10 min。1%琼脂糖凝胶电泳检测PCR产物。

使用DNA凝胶回收试剂盒（TaKaRa Biotechnology）对扩增的纤维素降解细菌16S rDNA进行纯化，1%琼脂糖凝胶电泳检测纯化结果。使用*Hha* I和*Afa* I两种四碱基限制性内切酶对目的片段进行联合酶切，酶切体系（5 μl）：ddH₂O 2.8 μl，*Afa* I酶（10 U μl⁻¹）0.1 μl，*Hha* I酶（10 U μl⁻¹）0.1 μl，缓冲液（10×T）0.5 μl，0.1% BSA 0.5 μl，PCR产物1.0 μl，37℃过夜。酶切产物经8%聚丙烯酰胺凝胶电泳，获得酶切带型图谱。在两种酶切图谱中均相同的克隆归为一个可操作分类单元（Operational Taxonomic Unit，OTU）。4个纤维素

表1 试验设计

Table 1 Design of the experiment

处理代号 Treatment code	试验处理 Experimental treatments
W _s S	玉米免耕播种、小麦翻耕播种，秸秆粉碎还田
W _{nt} S	玉米、小麦均免耕播种，秸秆粉碎还田
W _t	玉米免耕播种，小麦翻耕播种，秸秆不还田
W _{nt}	玉米、小麦均免耕播种，秸秆不还田

降解细菌16S rDNA基因文库的Shannon-Wiener、Simpson、Evenness和Richness多样性指数计算方法参照文献[18]。

1.5 测序与系统发育树构建

随机挑选1~2个代表每个酶切类型的克隆子进行测序，测序结果在NCBI(National Center Biotechnology Information)网站经BLAST分析，与Genbank数据库存在的序列进行同源性比对，用MEGA5.0(邻接法)构建系统进化树，根据序列在系统进化树的位置和遗传距离判定其系统发育地位，并进行典范对应分析。

2 结 果

2.1 耕作方式和秸秆还田对土壤纤维素降解细菌数量的影响

图1为土壤中纤维素降解细菌计数结果，显示出 W_tS 、 $W_{nt}S$ 、 W_t 和 W_{nt} 每克干土中所含纤维素降解细菌数量。对比可发现，耕作条件下，秸秆还田相

对秸秆不还田纤维素降解细菌数量增加了84%；免耕条件下，秸秆还田相对秸秆不还田纤维素降解细菌数量增加了116%；秸秆还田条件下，免耕相对耕作纤维素降解细菌数量提高了36%；秸秆不还田条件下，免耕相对耕作纤维素降解细菌数量提高了15%。由此可见，秸秆还田与免耕处理均不同程度提高了纤维素降解细菌数量。

2.2 耕作方式和秸秆还田对土壤纤维素降解细菌多样性的影响

从4种土壤样品中共筛选了425株纤维素降解细菌，采用Hha I和Afa I双酶切，共得到33个OTU，建立了4个纤维素降解细菌基因文库。图2显示4个文库的丰富度曲线均趋于平缓，其库容量均比较饱和，文库库容值在91.67%~95.28%范围内(表2)，表明所建立的基因克隆文库能比较完整地反映土壤纤维素降解细菌的群落结构。从文库多样性指数(表2)可以看出，4个文库的纤维素降解细菌多样性指数存在较大差异，秸秆还田加免耕处理多样性指数最高，且秸秆还田处理多样性指数高于

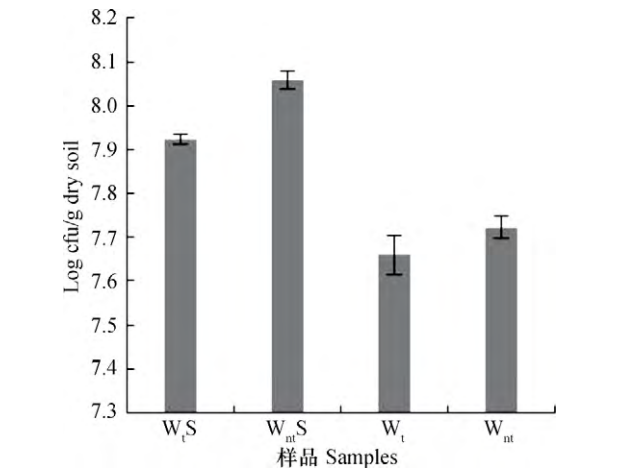


图1 土壤样品纤维素降解菌计数
Fig. 1 Counts of cellulose degrading bacteria in the soil samples

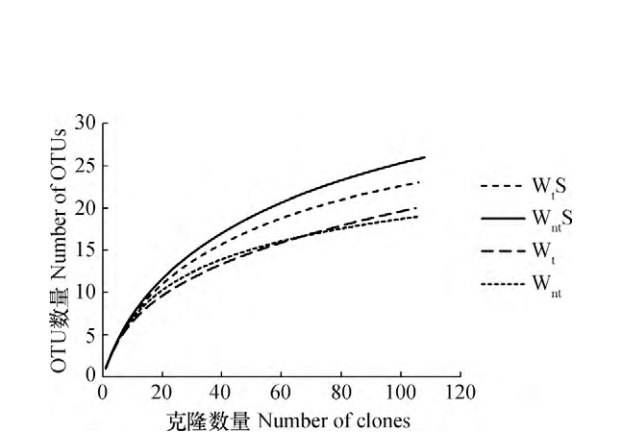


图2 四种土壤纤维素降解细菌基因文库丰度曲线
Fig. 2 Gene library abundance curves of the cellulose decomposing bacteria (CDB) in four types of soils

表2 四种土壤样品细菌基因文库的群落多样性结构指数

Table 2 Community diversity structural indices of the bacteria gene libraries in the four soil samples						
文库 Library	OTU种类 Type of OTU	覆盖值 Coverage C (%)	香农-威纳指数 Shannon-Wiener index	辛普森指数 Simpson index	均匀度 Evenness	丰富度 Richness
W_tS	23	93.40	2.67	0.905	0.573	4.72
$W_{nt}S$	26	91.67	2.79	0.914	0.596	5.34
W_t	20	92.38	2.44	0.881	0.524	4.08
W_{nt}	19	95.28	2.53	0.898	0.543	3.86

秸秆不还田处理，说明秸秆是否还田影响了土壤纤维素降解细菌多样性。此外，无论秸秆是否还田，免耕相对耕作更有利于提高土壤纤维素降解细菌多样性。

为了比较秸秆还田、免耕和对照组的纤维素降解细菌基因文库OTU类型差别，绘制了OTU类型文氏图（图3）。W_iS、W_{nt}S、W_t和W_{nt}处理分别获得OTU 23个、26个、20个和19个，表明秸秆还田处理中有更加丰富的纤维素降解细菌。在四种处理土壤中均存在的OTU共有7个，土壤经秸秆还田处理后，出现8个新OTU类型；经免耕处理后，出现3个新OTU类型，说明秸秆还田对土壤中纤维素降解细菌群落结构有明显影响，耕作方式对其也有一定的影响。

2.3 土壤纤维素降解细菌系统发育分析

将33个OTU类型的测序结果通过NCBI网站的BLAST分析比对，计算出四个处理的文库所含物种类群的百分组成（图4），并用Mega软件构建系统发育进化树（图5）。纤维素降解细菌16S rDNA同源性为80%~99%，33个OTU属于4个门、7个纲、11个目、15个科、20个属。耕作秸秆还田处理土壤中优势类群有*Sphingobium*、*Streptomyces*、

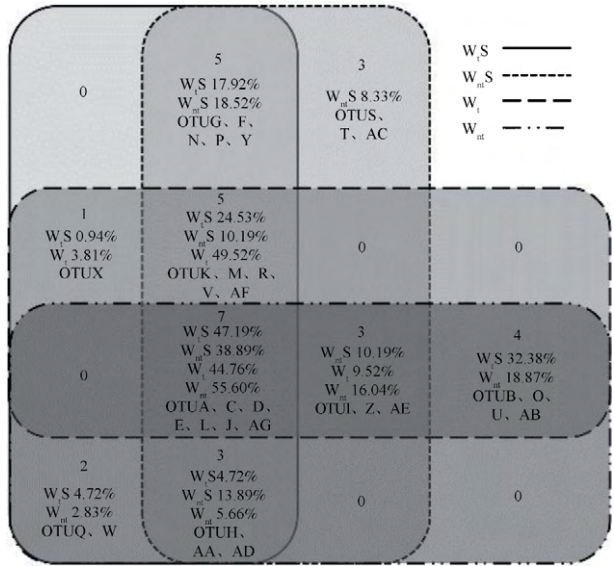


图3 反映4种处理土壤纤维素降解细菌基因酶切类型关系文氏图

Fig. 3 Venn diagram showing relationships between the genetic cleavage types of soil cellulose degrading bacteria in four soils

注：图中数字表示OTU个数，百分比表示克隆数占该文库的百分比，英文字母表示OTU种类

Note: The digits in the figure mean numbers of OTU, the percentages below percentages of clones in each corresponding library, and the English letters types of OTU

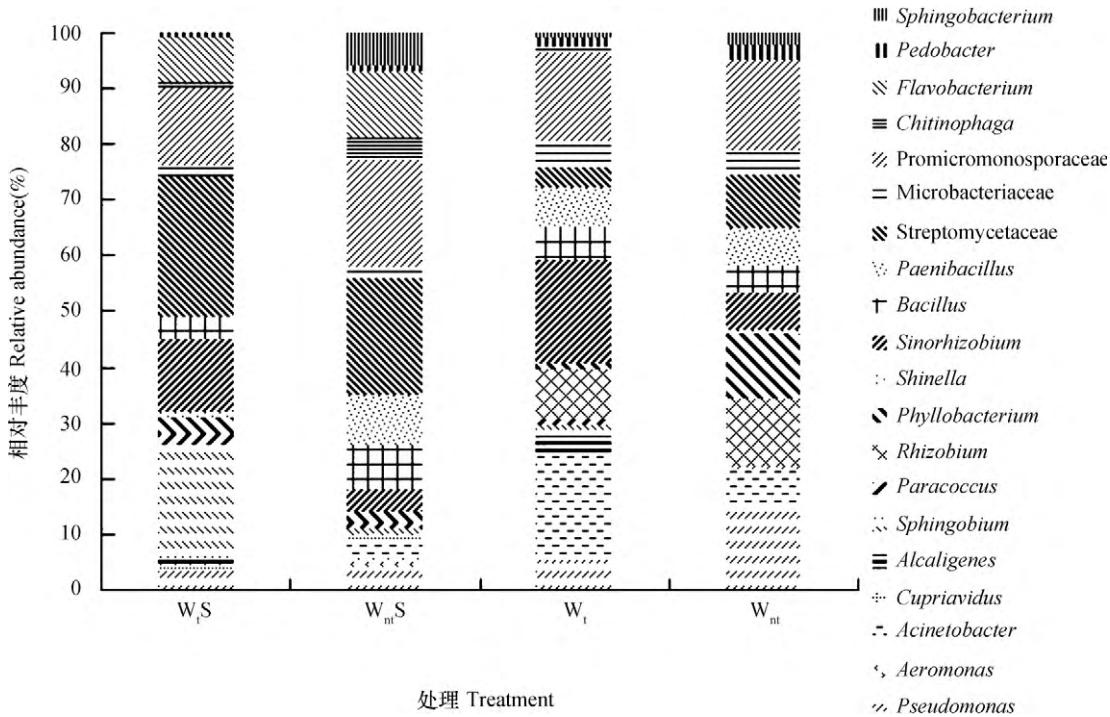


图4 纤维素降解细菌基因文库所含类群百分比

Fig. 4 Percentage of each group of CDB contained in the gene libraries

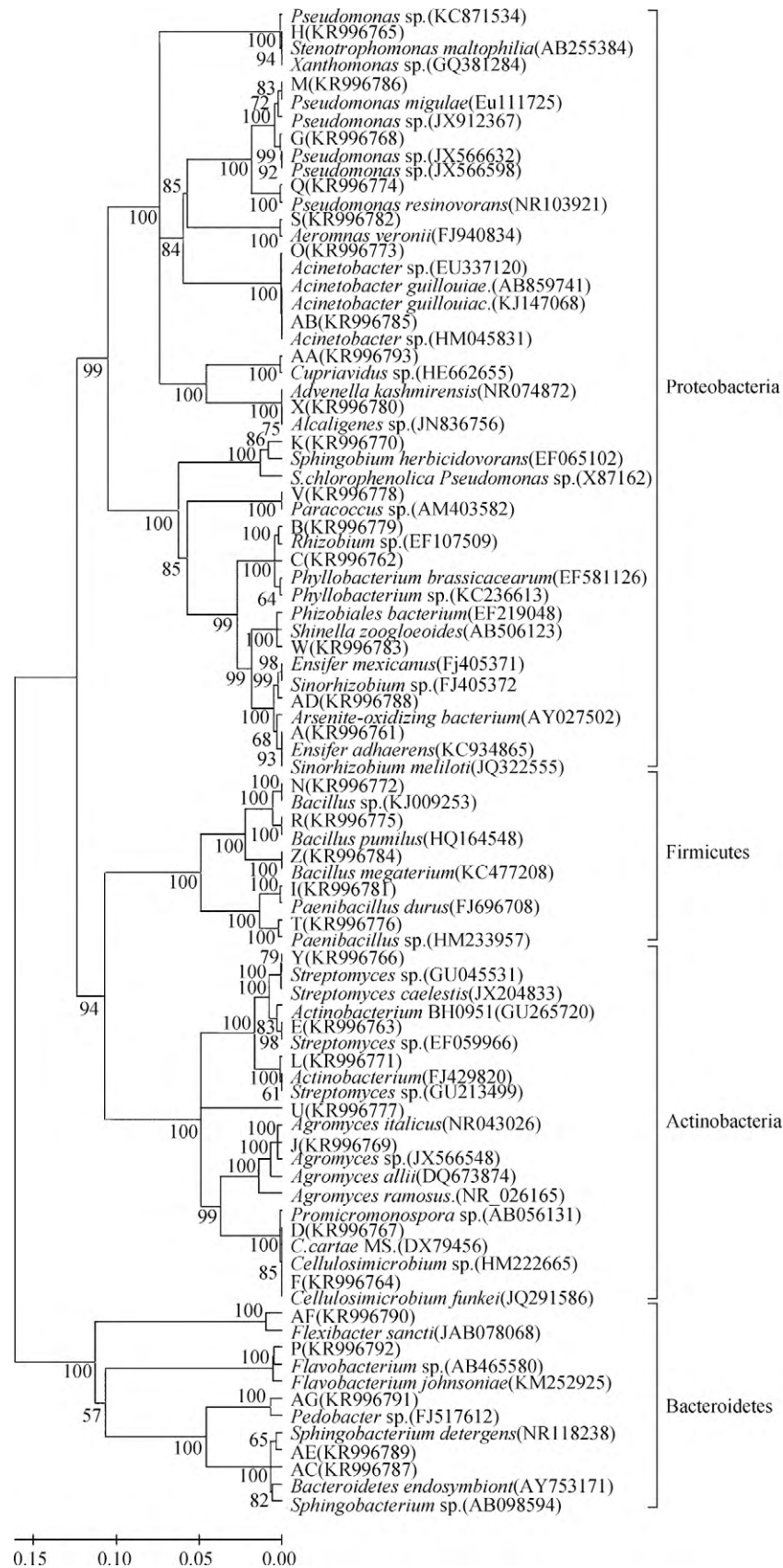


图5 基于4种土壤纤维素降解细菌16S rDNA序列的系统发育进化树

Fig. 5 Phylogenetic tree based on 16S rDNA sequences of the CDB in the four soils

*Sinorhizobium*和*Promicromonosporaceae*, 约占67.92%; 免耕秸秆还田处理土壤中优势类群为*Streptomyetaceae*、*Promicromonosporaceae*和*Flavobacterium*, 约占48.15%; 耕作秸秆不还田处理土壤优势类群为*Sinorhizobium*、*Promicromonosporaceae*和*Acinetobacter*, 约占54.29%; 免耕秸秆不还田处理土壤中优势类群为*Rhizobium*、*Phyllobacterium*、*Pseudomonas*和*Promicromonosporaceae*, 约占63.21%。秸秆还田土壤中*Streptomyetaceae*、*Flavobacterium*、*Sphingobium*相对丰度明显多于秸秆不还田处理土壤, 其中, *Flavobacterium*只出现在秸秆还田处理中且丰度较大, 说明秸秆还田有利于其生长繁殖, 它们在秸秆降解过程中发挥着重要作用。在免耕处理土壤中, *Pseudomonas*、*Phyllobacterium*、*Paenibacillus*、*Promicromonosporaceae*、*Sphingobacterium*相对丰度明显高于耕作处理土壤, 说明免耕可能有利于其生长繁殖。

本文得到的纤维素降解菌属于Proteobacteria、Firmicutes、Actinobacteria和Bacteroidetes 4个门, Proteobacteria为优势菌门, 有15个OTU, 所测序列共属于11个属。本研究还发现*Acinetobacter*、*Sphingobium*、*Sinorhizobium*、*Promicromonosporaceae*和*Streptomyetaceae*等纤维素降解菌丰度较高, 这些纤维素降解菌可能在土壤碳循环过程中有重要作用, 值得进一步研究。

2.4 环境因子与土壤纤维素降解细菌群落结构分布关系的典范对应分析

对测序序列与土壤理化性质进行典范对应分析, 考察不同处理以及各环境因子对纤维素降解细菌种群分布的影响(图6)。4种处理分属四个不同象限, 空间分布表现出显著差异, 表明4种处理纤维素降解细菌群落结构发生了较大变化。其中, pH、全磷、有机碳和全钾与免耕处理呈显著正相关性, 全氮、碱解氮、速效钾、速效磷和有机碳与秸秆还田处理呈显著正相关性, 速效磷与耕作秸秆还田处理有显著的正相关性。秸秆还田过程中, 秸秆中的氮、磷、钾等营养元素会改善土壤理化性质, 大量C/N比高的秸秆还可以增加土壤有机质含量, 调节土壤的C/N比, 为纤维素降解菌提供了基本的碳源和能源。免耕处理也会增加土壤有机碳和全氮的含量, 同样有助于纤维素降解菌的生长^[18-20]。

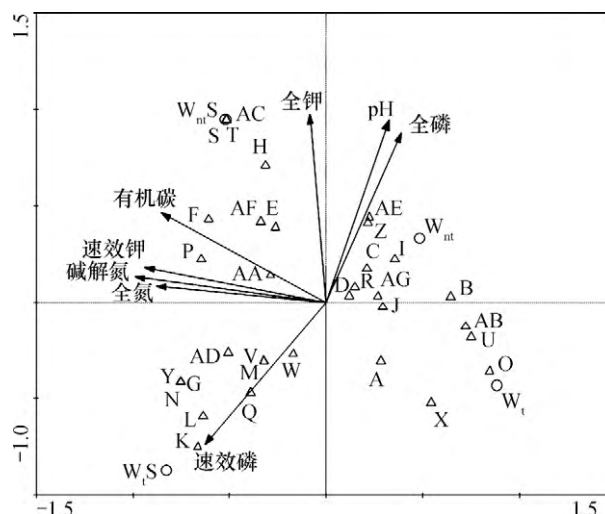


图6 环境因子与纤维素降解细菌群落结构的典范对应分析
Fig. 6 Canonical correspondence analysis of environmental factors and CDB community structure

3 讨 论

秸秆还田与免耕处理均不同程度提高了纤维素降解细菌数量, 且免耕配合秸秆还田能显著增加纤维素降解细菌数量, 这与目前研究结果基本一致^[21], 李晓莎等^[22]发现秸秆还田结合保护性耕作在冬小麦/夏玉米轮作区能显著提高微生物生物量碳和微生物活性。纤维素降解菌是土壤有机残体分解的中心环节, 其数量和分解强度反映了土壤微生物对有机残体分解的速度和程度, 直接关系着土壤有机质的形成与积累^[23]。免耕配合秸秆还田使纤维素降解细菌数量增加, 可能是由于免耕减少了对土壤的扰动, 增加了底物利用率; 免耕和秸秆还田还增加了土壤有机质含量^[24-26], 土壤有机质与微生物量碳有显著的正相关关系^[27], 因而增加了纤维素降解细菌数量, 有助于纤维素降解与养分释放; 免耕措施避免了土壤结构的破坏, 增加了植物根系在土壤中的含量, 产生的根系分泌物使土壤养分含量增高。本实验站地点的其他研究也表明, 秸秆还田和保护性耕作对提高土壤有机碳库, 尤其对土壤0~10 cm土层有机碳、氮的累积具有积极的作用^[28-29]。

对4种不同处理土壤中纤维素降解细菌群落结构多样性的分析表明, 秸秆还田处理OTU数量明显高于秸秆不还田处理, 免耕与否对OTU数量影响次之, 由此可见, 秸秆还田与免耕能改变土壤纤维素降解细菌群落结构, 提高土壤纤维素降解

细菌多样性。系统发育分析表明,所有OTU属于Proteobacteria(变形菌门)、Firmicutes(厚壁菌门)、Actinobacteria(放线菌门)和Bacteroidetes(拟杆菌门),占总克隆数的比例分别为45%、11%、34%和10%。对森林土壤中纤维素降解菌种类的研究中,Talia等^[30]通过CMC培养基富集纤维素降解菌获得52个克隆,Proteobacteria、Actinobacteria和Firmicutes分别占总克隆的90%、6%和4%,可以发现,自然界中,变形菌门和放线菌门存在较多的纤维素降解菌。

通过序列的比对分析,我们发现纤维素降解菌16S rDNA同源性高低相差悬殊,表明这类功能微生物有广泛的系统发育分布,寻找保守序列设计通用引物是研究纤维素降解细菌的一个难点。对微生物组成与土壤理化性质进行了典范对应分析,结果表明,免耕与秸秆还田对土壤多个理化因子有较大影响作用,多个环境因子对纤维素降解细菌的种群分布也存在较显著的影响。

秸秆还田与免耕具有诸多优势,秸秆还田不仅减少了其焚烧所带来的环境污染,并且提高了土壤有机质含量,促进团聚体形成,增加了微生物活性和生物固持力,改善了土壤质量和生态环境,进而促进作物的生长发育。免耕节省了大量的人力和物力,具有良好的经济效益。本研究明确了纤维素降解细菌数量和种类的变化特征,为深入理解免耕与秸秆还田条件下土壤质量演变的微生物学机理提供科学依据。

4 结 论

小麦和玉米轮作是黄淮海平原广泛分布的种植模式,潮土是该地区的典型土壤,通过长期定位试验小区研究表明,免耕和秸秆还田能够增加土壤纤维素降解细菌的数量,改变其群落结构组成,提高其群落结构多样性。因而,作为可持续农业的关键技术,免耕和秸秆还田通过增加微生物数量和种类来提高土壤质量,有一定的理论意义和巨大的推广潜力。

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Effects of Tillage and Straw Mulching on Diversity of Cellulose-decomposing Bacteria in Fluvo-aquic Soil

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Abstract 【Objective】 No-tillage and straw mulching are two important techniques in soil building. It is, therefore, of great significance to understand effects of the two on diversity of the cellulose-decomposing bacteria (CDB) community in the soil. To this end, analyses were done of effects of long-term no-tillage and straw mulching on diversity of CDB, microbial community structure, and richness and dominance of CDB, so as to provide a theoretical basis for the study and demo-extrapolation of the technique of conservation tillage. 【Method】 A long-term experiment, designed to have four tillage treatments, i.e. tillage with mulching (W_tS), no-tillage with mulching ($W_{nt}S$), tillage without mulching (W_t) and no-tillage without mulching (W_{nt}), was conducted at the State Experimental Station for Agro-Ecology in Fengqiu, Henan Province. Soil samples were collected from the plots of the four treatments, incubated in CMC-Na Congo red medium for counting of CDB and analyzed with PCR-RFLP for effects of no-tillage and straw mulching on CDB community. 【Result】 The results of plate counting show that the population of CDB was 8.40×10^7 (W_tS), 11.4×10^7 ($W_{nt}S$), 4.57×10^7 (W_t) and 5.27×10^7 (W_{nt}), indicating that both No-tillage and straw mulching increased the number of CDB to a varying extent. Out of the soil samples collected from the four treatment plots, a total of 425 strains of CDB were isolated, 4 CDB gene libraries built based on treatment, and a total of 33 OTUs obtained. The number of OTUs of CDB in the treatments range from 19 to 26, among which 7 were found common in all the 4 treatments. 8 particular in the treatments with straw mulching soil and 3 in the treatments of no-tillage, which indicates that the practices of straw mulching and no-tillage did have some effects on CDB community structure in the soil. The 4 clone libraries varied sharply in CDB diversity index, which was the highest in Treatment $W_{nt}S$, and higher in the two treatments with straw mulching than in the two without straw mulching, which indicates that compared with tillage, no-tillage is more conducive to diversity of the CDB in the soil. Phylogenic analysis shows that the CDB in the soils varied in the range of 80% ~ 99% in 16S rDNA homology. The 33 OTUs could be sorted into 4 phyla, 7 classes, 11 orders, 15 families and 20 genera. Proteobacteria is the dominant phylum, composed of 15 OTUs, which could be sorted into 11 genera. In Treatment W_tS , *Sphingobium*, Streptomycetaceae, *Sinorhizobium* and Promicromonosporaceae are the dominant groups, accounting for 67.92%, in Treatment $W_{nt}S$, Streptomycetaceae, Promicromonosporaceae and *Flavobacterium* are, accounting for 48.15%, in Treatment W_t , *Sinorhizobium*, Promicromonosporaceae and *Acinetobacter* are, accounting for 54.29%, and in Treatment W_{nt} , *Rhizobium*, *Phyllobacterium*, *Pseudomonas* and Promicromonosporaceae are, accounting for 63.21%. The treatments with straw mulching are significantly higher than the treatments without straw mulching in relative abundance of Streptomycetaceae, *Flavobacterium* and *Sphingobium*, which indicates that straw mulching is conducive to growth of these bacteria, which play an important role

in straw decomposition. And the treatments of no-tillage are apparently higher than the treatments of tillage in relative abundance of *Pseudomonas* , *Phyllobacterium* , *Paenibacillus* , *Promicromonosporaceae* and *Sphingobacterium* , which indicates that no-tillage is conducive to growth of these bacteria. Canonical correspondence analysis shows that great changes have taken place in CDB community structure in all the four treatments , that pH , TP , organic carbon and TK is significantly and positively related to no-tillage and that TN , analyzable N , readily available K , readily available P and organic carbon is significantly and positively related to straw mulching. 【Conclusion】 It is quite clear that no-tillage and straw mulching can significantly raise the number and diversity of cellulose-decomposing bacteria in the soil , and alter cellulose-decomposing bacteria community structure. Conducted as key techniques for sustainable agriculture to improve soil quality by increasing the number and variety of microorganisms , no-tillage and straw mulching or incorporation do have some theoretical significance and enormous potential for extrapolation.

Key words No-tillage ; Straw mulching ; Cellulose-decomposing bacteria ; Diversity ; PCR-RFLP

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