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棉花长期连作对新疆土壤细菌群落结构的影响

张 伟[1,2,*],陈一峰[2]
(1. 新疆师范大学生命科学学院,乌鲁木齐 830046; 2. 新疆农业科学院微生物应用研究所, 乌鲁木齐 830000)

摘要:新疆部分棉区发生连作障碍后与其他地区有所不同,能自发恢复并长期保持高产、稳产,为了查明该类棉田土壤细菌群落结构在连作障碍发生及自发恢复整个过程中的演替规律。以未开垦土地作为对照,利用 16S rRNA-PCR-DGGE(polymerase chain reaction-density gradient gel electrophoresis)法对比研究了新疆阿克苏棉区分别连作 1、3、5、10、15 和 20a 棉田 1—30 cm 深度土壤细菌群落结构组成。结果表明未开垦土地细菌多样性指数丰富度最高,多样性和均匀度指数最低。随着棉花连作年限的延长,土壤细菌群落丰富度指数不断下降,而多样性和均匀度指数逐渐增大。当连作年限继续延长至 10a 后各指数出现恢复或趋于达到一个新的相对稳定状态。聚类分析显示 7 个样品分别聚为 3 簇,其中连作 3a 的样品差异最大,相似度仅有 44%,而连作 10a 后的样品和对照较为相似。主成分分析也有类似的结果。对比回收的部分序列显示,序列间相似性在 88%以上,分属于 *Microbacterium*、Uncultured *Chloroflexi* bacterium、TM7 *Phylum* sp. Canine、*Flavobacteria* 等 4 个不同菌属。分析认为棉花长期连作对该地区土壤细菌群落结构组成影响很大,但随着连作年限延长至 5a 以后,细菌群落结构组成能自发趋于稳定和回升。此外,对比棉田细菌群落结构整体变化规律和棉花产量的增减及病虫害发生规律发现,在棉花长期连作过程中两者有很强的关联性。

关键词:棉田土壤;细菌群落;16S rRNA-PCR-DGGE;聚类分析;主成分分析

Analysis of the bacterial communities in continuous cotton fields of Xinjiang Province

ZHANG Wei[1,2,*], CHEN Yifeng[2]
1 *College of Life Science, XinJiang Normal University, Urumqi* 830046, *China*
2 *Institute of Microbiology, Xinjiang Academy of Agricultural Sciences Urumqi* 830000, *China*

Abstract: Long-term continuous cropping of cotton has caused dramatic soil-borne diseases in many places, leading to substantial agricultural losses. However, in some areas of Xingjang Province, the obstacles caused by continuous cotton cropping can spontaneously restore and maintain high yields of cotton for many years. To analyze the variable spectrum of soil bacterial communities and changes in the community structure in these spontaneously restored fields during cropping, soils at depths from 1 to 30 cm were sampled from cotton fields with a history of 0, 1, 3, 5, 10, 15 or 20 years of cotton cropping in the Akesu region of Xinjiang Province. The bacterial communities in these samples were studied using 16S rRNA-based polymerase chain reaction-density gradient gel electrophoresis (PCR-DGGE) with samples from uncultivated land as a control. Bacterial community diversity indices including the Shannon-Wiener diversity (H), Abundance index (S) and Evenness index (E_H) were compared among these samples. Samples from uncultivated land had relatively high levels of the richness indices but both the Diversity and Evenness Indices were at lower levels. With increasing years of cotton cropping, both the bacterial Diversity and Evenness Indices increased, whereas the richness indices showed a general

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* 通讯作者 Corresponding author.E-mail: zw0991@sohu.com

decrease. However, after 10 years of continuous cropping all these indices were restored to their original values or reached a relative stable level. Cluster analysis of DGGE fragments indicated that the seven samples were clustered into three branches: fragments from samples under successional cropping for 0, 10 and 20 years formed one small branch with a similarity of approximately 50%; fragments from successional cropping for 1, 5 and 15 years formed another branch with a similarity of 53%; and the last branch comprised fragments from successional cropping for 3 years with a similarity of 44%. Principal component analysis (PCA) showed that all of the samples were statistically correlated with the major component and fluctuated on the right of the major component between the positive and negative axes of the second principal component. Both cluster analysis and PCA results suggested that, compared to those from original uncropped fields, the bacterial community structure showed the most variation in samples from the field of 3-year cropping, whereas similar patterns of bacterial community structure were found between samples from fields of 10 years of cotton cropping and those from uncropped fields. Nineteen clones were sequenced from each band and among them one sequence was selected and submitted to GenBank (accessory no. JN572545-JN572563). By aligning with the GenBank database, all sequences from DGGE were classified into four groups: *Microbacterium*, Uncultured *Chloroflexi* bacterium, TM7 *Phylum* sp. canine, and *Flavobacteria*. Further analysis demonstrated that the isolated V3 sequences showed a homology of 88%—100% to known sequences in GenBank and 47% of the sequences belonged to bacteria which were not cultured. No microbial data were correlated with soil-borne plant diseases of cotton. The study demonstrated that the age of cotton fields had significant effects on soil bacterial diversity. Continuous cotton cropping exerted significant influences on the community structure of soil bacteria in Xinjiang Province, with an initial suppression effect on bacterial diversity. However, the bacterial community reached a stabilized or even increased level compared with its original state after 5 years of continuous cropping. In addition, correlations between variations in the bacterial community structure at a depth of 1—30 cm and the yield of cotton and pest disease attacks were also found in this study.

Key Words: cotton field soils; bacterial communities; 16S rRNA-PCR-DGGE; cluster analysis; principal component analysis

新疆是我国最早种植棉花的地区之一,也是目前我国唯一的长绒棉种植基地。由于新疆地区具有日照充足,降水稀少,空气干燥等多种适于种植棉花的自然环境条件,自 20 世纪 80 年代以来新疆棉花的种植面积不断扩大,至 2012 年新疆棉花在种植面积、总产量、平均亩产等 7 项指标上已连续 21a 位居全国首位[1-2]。与此同时,由于新疆农作物种类单一,长期以来棉花连作现象严重,尤其是主产区,棉花种植面积占到 95%以上,休作、轮作几乎是不可能的。连作障碍是植物和土壤两个系统内部诸多因素综合作用的结果[3],土壤微生物群落结构的稳定对维持土壤系统的健康和质量非常关键[4],对植物生长具有重要作用[5]。土壤微生物群落结构的变化会直接影响土壤功能的发挥[6]。与其他地区作物连作一样,新疆棉花连作也带来了病虫害持续加重[7]、土壤中农药、化肥、地膜污染长期积累等生态问题[8-9]。但值得关注的是部分地区的棉花随着连作年限的继续延长,发生连作障碍后只需施用较少的农药、化肥,连作障碍现象会自发的缓解并长年保持高产、稳产,因此连作年限达到 30a 以上,这一现象引起人们的关注。

新疆棉花主产区存在着棉花连作时间跨度从 0—30a 的各类棉田,棉花长期的种植已经形成了独特的棉田土壤微生物群落[10-11]。这些微生物参与土壤物质转化过程,在土壤形成、肥力演变、农业污染物的降解和土壤结构的形成与改良等方面起重要作用,但长期的棉花连作对于微生物群落结构带来的影响和微生物群落结构的变化与棉花连作后产量的下降及病虫害发生规律之间的关系还不清楚。PCR-DGGE 技术近些年在微生物生态领域有广泛的应用[12-16],本文利用该技术着重研究随着棉花连作年限的延长,土壤中细菌群落在组成、多样性和演替等方面发生的变化,试图从土壤微生物生态的角度解释新疆棉花长期连作后连作障碍的发生及自发消

除,以及该地区棉花种植过程中病虫害的发生规律与土壤微生物群落结构演替之间的联系。

1 材料和方法

1.1 样品的采集

采样时间和地点: 2011 年 8 月于新疆阿克苏高产棉区,选择棉花连作年限为 0(尚未开垦的土地)、1、3、5、10、15 和 20a 的土地分别收集土样。方法:土钻垂直打下 30 cm(耕作层)深度取土,相同连作年限的棉田选择不同的 5 处采集等量样品混合后作为一个土样,合计为 7 个土样。采样范围在(E 80°16′77″—24′45″,N 41°07′47″—24′85″)之间。土样采集后低温保存带回实验室,-80 ℃储存使用。

1.2 样品总 DNA 的提取和纯化

DNA 的提取参照 Zhou 等的 SDS based DNA extraction 法[17-18],唯一改动之处是,在氯仿、异戊醇抽提前多加了一步等体积的酚、氯仿和异戊醇抽提。DNA 的纯化按 Moreira 的方法[19],每一 DNA 样品做 3 个重复,等量混合后备用。

1.3 16s rRNA 片段的 PCR 扩增

第一套 PCR 用细菌的通用引物 For/Dev 扩增差不多全长的 16s rRNA[20](表 1)。25 μL 反应体系:2.5 mmol/L 的 dNTP 2.8 μL,2.5 μL 10x Buffer,1u Taq 酶,引物各 3 pmol。反应程序:94 ℃,5 min;94 ℃,60 s;55 ℃,45 s;72 ℃,60 s,共 30 个循环;72 ℃,5 min 后稳定在 4 ℃。

第二套 PCR 用 F341GC/R534 引物扩增细菌 16 s rRNA v3 区[21](表 1)。25 μL 反应体系:稀释 100 倍的第一套 PCR 产物 1 μL,2.5 mmol/L 的 dNTP 2.8 μL,2.5 μL 10x Buffer,1 u Taq 酶,引物各 5 pmol。反应程序:94 ℃,5 min;94 ℃,60 s;55 ℃,45 s;72 ℃,60 s;共 30 个循环:72℃,5 min 后稳定在 4 ℃。

1.4 PCR 产物的 DGGE 电泳

用 D-code System 电泳仪(Bio-Rad 公司)进行 DGGE 电泳分离。制备变性梯度凝胶,使聚丙烯酰胺凝胶(Polyacrylamide gel electrophoresis,PAGE)浓度为 6%—8%,变性梯度 30%—70%(7mol/L 尿素和 40% 甲酰胺为 100% 变性),电泳缓冲液为 1x TAE,25 μL 第二套 PCR 产物在 60 ℃,150 V 条件下电泳 4 h,取出后用硝酸银法染色[22]。FR-200 紫外与可见分析装置(复日科技)下进行拍照。

表 1 用于 16s rRNA 扩增的引物

Table 1 Primer for 16s rRNA amplication

引物名 Primer	16s rRNA 位点 16s rRNA target	引物序列 Primer sequence(5′—3′)	参考文献 References
For	9—27	GAGTTTGATCCTGGCTCAG	[20]
Dev	1541—1525	AGAAAGGAGGTGATCCAGCC	[20]
F341-GC	341—359	GC-CCTACGGGAGGCAGCAG	[21]
R534	534—518	ATTACCGCGGCTGCTGG	[21]

在 5′端加入了一个富含 GC 序列的 GC 夹:CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G

1.5 数据处理

用 Quantity One v4.62 软件对 DGGE 图谱进行数字化、标准化后,得到一个记录 DGGE 胶中条带迁移位置和亮度的数字化矩阵。系统自动依据条带的有无按照非加权成对算术平均法(Unweighted Pair-Group Method with Arithmetic,UPGMA)对各泳道土壤样品进行聚类和相似性比较。用 Gel-Pro analyzer、SPSS16.0 和 Excel 软件直接读取 DGGE 指纹图谱条带信息,以代表每一细菌种群的条带在每一泳道中亮度峰值的百分含量为重要值对样品进行主成分(Principal component analysis, PCA)和相关方差分析等工作。用 Shannon-Wiener 指数(H)、丰富度(S)和均匀度(E_H)来评价土壤细菌群落的多样性,其算式为:

$$H = \sum_{i=1}^{s} p_i \ln p_i$$

$$E_H = H/H_{\max} = H/\ln S$$

式中,p_i为某一条带的强度与同泳道中所有条带总强度的比值,S 为每一泳道总的条带数[23]。根据泳道的 p_i值进行 PCA 分析。

1.6 特异序列的回收及信息比对

选择并回收 DGGE 凝胶上 0a 样品中所占灰度比较大的条带和连作样品中出现的特异条带。回收和克隆参考 Daniela 的方法[24]。对回收的序列上传

至 GenBank,获得的序列号是 JN5725- 63。通过 BLAST 查询及 CLUSTAL X2.0、MEGA4.0 软件处理构建相关序列进化树。

2　结果与讨论

2.1　连作对棉田细菌多样性的影响

细菌 16s rRNA 的 PCR-DGGE 图谱显示条带数较多,可见各样品中细菌多样性都很丰富。其中不同连作年限土样分离出有相同的带也有不同的带,但带的多少及光密度都发生了较大变化(图 1)。通过软件进一步数字化处理后得到表示土壤细菌多样性的 Shannon-Wiener 指数(H)、丰富度(S)和均匀度(E)指数。由数据看出,香农多样性指数未开垦地最低为 2.98,随着连作年限的增加,细菌多样性指数先增加后减少,其中连作 5—15a 的数值比较稳定。丰富度未开垦土地最高为 69,连作后都有所降低,最低为未开垦地为 46。均匀度是未开垦地最低为 0.668,连作后有所增加,其中 10a 的最高为 0.887(表 2)。由此可知棉花长期连作使土壤细菌群落结构类型发生了很大改变[25-28]。首先,未开垦土样香农多样性和均匀度指数最低,但其丰富度最高,分析原因可能是原生态土壤中有机质和水分等含量较少不利于细菌的大量繁殖,但细菌群落结构组成较丰富[12]。其次,棉花的种植使土壤中细菌多样性和均匀度增大,而丰富度下降,尤其是连作前 5a 各指数的变化较大,而随着连作年限的继续延长,各指数的变化趋缓。分析原因可能是棉花的种植给土壤输入较多的有机质等营养物质导致细菌的大量繁殖,而某些种类的细菌由于环境的不利改变而消失[7]。

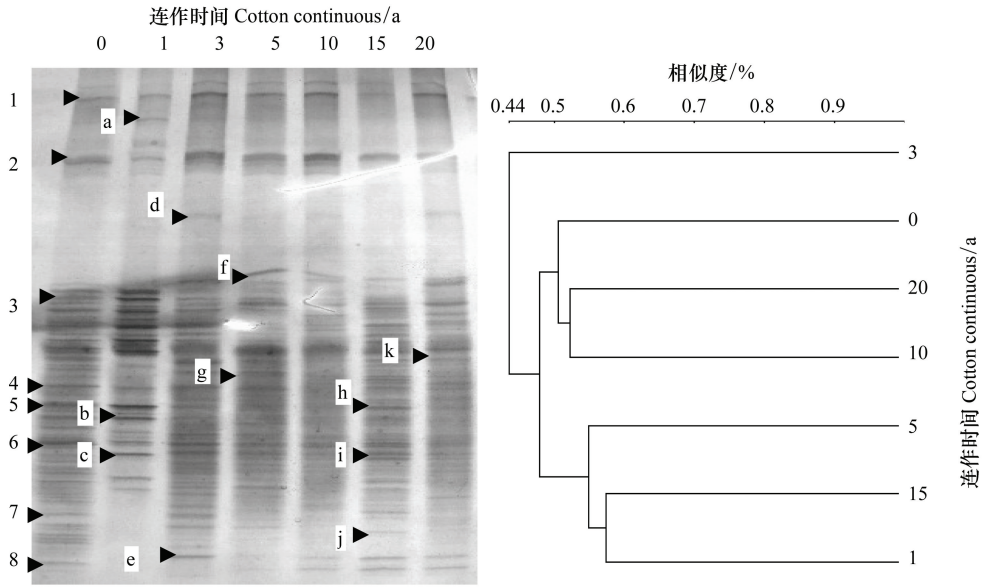

图 1　棉花连作 0、1、3、5、10、15、20a 的土壤细菌 16s rRNA DGGE 图谱及聚类分析

Fig.1　DGGE patterns and cluster analysis of 16s rRNA from 0, 1, 3, 5, 10, 15 and 20 years cotton continuous cropping field, respectively

表 2　各样品细菌 16s rRNA DGGE 图谱多样性指数(H)、均匀度(E)和丰富度(S)指数

Table 2　Shannon-Weaver diversity (H), eveness index (E) and abundance index (S) of the cotton soils bacterial community with difference continuous cropping years

样品 Number	样地 Sample	香农-威纳指数 Shannon-Wiener diversity	丰富度指数 Abundance index	均匀度指 Eveness index
1	连作 0a	2.98±0.13b*	69±4.5a	0.668±0.06a
2	连作 1a	3.12±0.14b	46±0.6a	0.841±0.03b
3	连作 3a	3.41±0.17a	67±0.5a	0.811±0.02b
4	连作 5a	3.28±0.13b	56±3.5b	0.815±0.01b
5	连作 10a	3.44±0.11a	48±4.0b	0.887±0.07a
6	连作 15a	3.65±0.10a	67±0.5a	0.868±0.02b
7	连作 20a	3.06±0.09a	48±0.5b	0.790±0.04a

* a, b 表示在 $P<0.05$ 水平下有显著差异

2.2 连作对棉田细菌群落结构组成的影响

聚类分析结果表明:土壤中细菌群落结构组成发生了较大的变化。其中,连作 1、5 和 15a 的聚为一小类,相似度达 55%以上。连作 0、10 和 20a 的聚为另一小类,相似度达 50%以上。而连作 3a 的最后才聚到一起,相似度只有 44%(图 1)。可见连作 3a 的土样细菌群落结构变化最大,而随着棉花连作年限的延长,细菌群落结构呈现反复波动并有恢复的趋势。对 DGGE 图谱各泳道每个条带的 P_i 值进行主成分分析,结果表明,第一主成分方差贡献率达 55.37%,第二主成分方差贡献率达 15.5%,积累方差达 70.87%。在这两个主成分为坐标轴构建的二维坐标系中,不同连作年限样品与对照相比都发生了明显的位置变化,但又明显被分为两组,其中连作 5、10、和 20a 样品与对照较为一致,而连作 1、3 和 15a 样品变化趋势较一致,其中棉花连作 3a 对土壤细菌群落组成影响最大,这点和聚类分析结果一致(图 2)。总体分析原因可能在外界环境发生长期一致的改变后,土壤细菌群落为应对外界环境的变化自发的调整结构类型,表现在反复波动中逐渐恢复并稳定下来,同时显示出一定的稳定性[25]。由此可见棉花的长期种植不仅改变了原土壤细菌群落的微环境,并影响了土壤细菌群落结构组成。

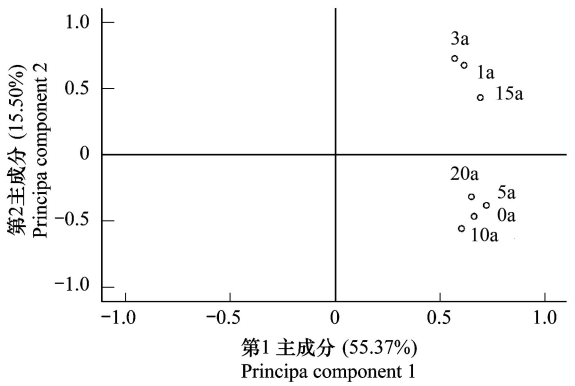

图 2 样品细菌群落结构主成分分析

Fig.2 Principal component analysis of the monoculture cotton soil bacterial with different years

2.3 序列同源性分析

条带 1—8 是 0a 样品强度较大的,条带 a—k 是棉花连作不同年限后新出现的强度较大的(图 1)。这 19 个条带之间同源性在 88%—100%之间。部分序列与已知的 *Microbacterium* insulae、*Devriesea* agamarum、*Atopobium* rimae 等微生物同源性达到 100%,并隶属于多个属。查阅已知的与 DGGE 凝胶上回收条带序列同源性最近的微生物信息,都属于土壤中常见的类型(进化树结果)(图 3)。由于土壤细菌数量和种类繁多,DGGE 凝胶上出现的条带很多,而每一条带代表的是一类细菌[26],虽然回收了部分特异的条带,但测序后并没有发现有导致棉花病虫害的微生物信息,至于哪些菌群发生的改变会影响棉花产量和病虫害的发生还有待深入研究[26]。

2.4 连作棉花发病规律、产量变化与土壤细菌群落结构变化的关系

耕作土中细菌群落的结构组成和分布特征受土壤理化性质、当地水文气候等的影响[27],此外耕作制度对土壤细菌结构组成的影响也是非常重要的[28-29]。目前关于农作物连作对土壤细菌群落组成的研究比较多,但还是不能完全清楚土壤微生物与作物之间的复杂相互关系。新疆作为我国长绒棉种植基地,与其他地区作物连作一样,起初棉花连作也带来了病虫害加重、产量下降等负面影响[30],但随着连作年限的继续延长,连作障碍的现象能自发缓解[7]。针对这一现象本文研究了 0—20a 棉花连作过程中土壤细菌群落结构组成的变化规律。在参考当地农技部门记载的数据和走访棉花种植户后发现:棉花连作障碍的发生与土壤细菌群落结构的变化规律在时间上比较一致[31-32]。比如,连作 3a 是产量较低、病虫害较重的阶段,而土壤细菌群落结构变化也最大。随着连作年限延长至 5a 之后各种负面影响趋于减缓,10a 之后甚至出现恢复的迹象。研究后发现土壤细菌群落组成也在这个阶段出现稳定及恢复的趋势。但由于土壤细菌组成和功能的复杂性,对于土壤细菌群落组成与农作物之间的互作关系了解较少,因此还需要做更深入的研究才能更好的给当地棉花连作造成的土传病虫害防治及耕作管理制度的改善提供指导[33]。

3 结论

首先:与利用其他方法的相关研究报道有相似之处[33-34],都发现棉花单一作物长期连作对本地区土壤细菌群落结构组成及功能有重大影响,表现在随着连作年限的增加,土壤细菌结构组成发生反复波动。当连作年限超过 5a 后,土壤细菌群落组成逐渐趋于稳定,并与原生态土壤细菌结构组成有一

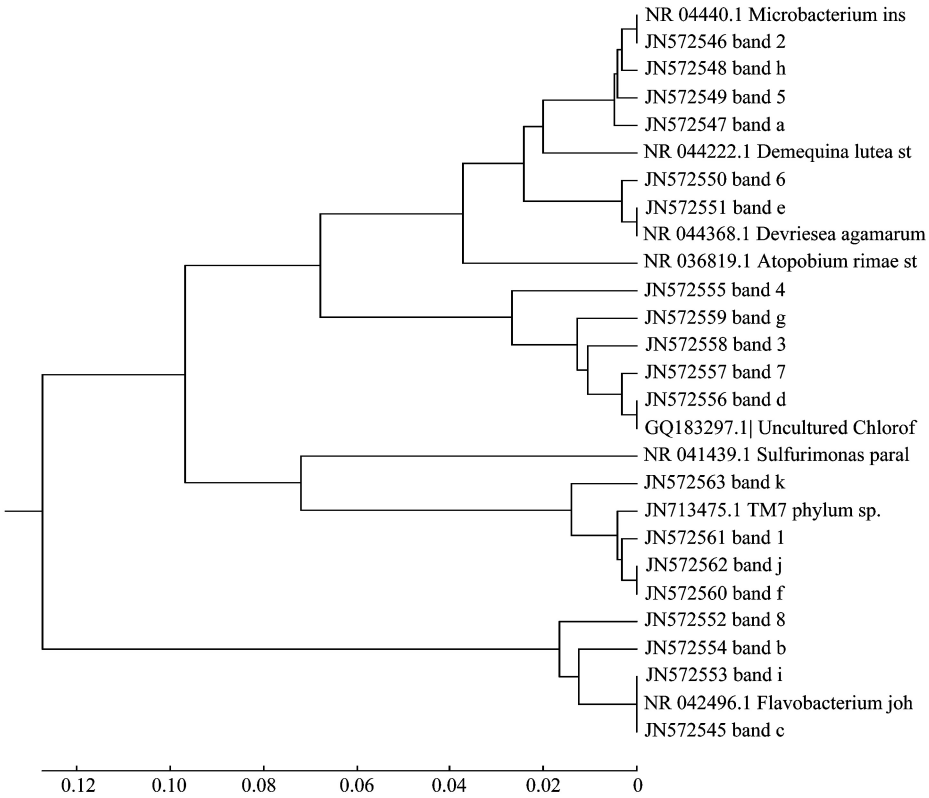

图 3　对回收的序列和相似性序列绘制系统树

Fig.3　The closest sequence match of known phylogenetic affiliation to band sequences extracted from DGGE gel

定的相似性。其次,长期一致的外界胁迫可以使土壤微生物群落结构组成发生适应性改变,但在某些特殊的环境下也有可能形成新的、稳定的、健康的、适合作物长期种植的结构类型。此外,在长期的连作过程中,土壤会发生“细菌型”向“真菌型”的转变[35],因此也有必要研究土壤真菌的结构组成及多样性随着连作年限延长发生的变化。

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