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Original Research

Accumulation and Distribution of Cadmium in Flue-Cured Tobacco and Its Impact on Rhizosphere Microbial Community

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Abstract

To explore the accumulation and distribution of Cadmium (Cd) in flue-cured tobacco and the effect on soil microbial community structure in the rhizosphere, pot experiments were conducted with different treatment levels of Cd (0, 2, 4, 8, and 16 mg·kg^{-1}). The Cd accumulation in different organs of flue-cured tobacco was analyzed, and the bacteria and fungi community structures in the rhizosphere were examined using PCR-DGGE fingerprinting with universal bacteria and fungi primers. Results showed that the order of Cd contents in different organs of flue-cured tobacco was: leaf > stem > root. Increasing Cd concentration in the pot soil elevated Cd contents in different organs of flue-cured tobacco. As to the soil microbial community, the bacterial fingerprinting bands in rhizosphere with 4 mg·kg^{-1} Cd level were significantly less than other treatments. Some bacteria disappeared with increasing soil Cd concentrations. Nevertheless, some special bacteria apparently had a strong restoration capability in the rhizosphere at a high Cd contamination level (16 mg·kg^{-1}). Among the 8 clusters of bacterial communities identified by the sequencing, the *Bacterium ellin*, *Acidobacteria bacterium ellin*, and *Mycobacterium* had strong resistance and adaptability to Cd contamination of different concentrations, whereas the *Leptolyngbya* had strong adaptability to Cd contamination of moderate concentration, which could become the dominant population at this level. The fungi community diversity in the rhizosphere with different concentrations of Cd was significantly different from the pattern of bacteria. Some fungi appeared with the increase of Cd concentration, but some fungi disappeared at the moderate Cd concentration (4 mg·kg^{-1}). Among the 4 clusters of fungal communities identified by the sequencing, the *Fusarium* and unknown species were the dominant species in the fungi community, which existed in all treatments and had strong adaptability to different Cd concentrations. The *Acremonium sclerotigenum* was sensitive to Cd contamination and disappeared at Cd concentrations greater than 2 mg·kg^{-1}, but *Rhizopus* appeared at Cd concentrations greater 8 mg·kg^{-1}, suggesting its preference for high Cd concentrations. Flue-cured tobacco had a strong ability to absorb and accumulate Cd. A proper concentration of Cd might have a positive effect on the soil bacteria and fungi community structure of flue-cured tobacco rhizosphere.

Keywords: accumulation and distribution, cadmium contamination, flue-cured tobacco, microbial community structure, rhizosphere

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Introduction

Heavy metal contamination is one of the major environmental problems in many parts of the world. Heavy metal pollution has been shown to adversely affect ecosystems and is potentially harmful to biota. Among the heavy metals, cadmium (Cd) is commonly associated with soil pollution, and considered to be particularly toxic to and responsible for significant decreases in biological activities in soils [1-3]. Cd is a non-degradable metal pollutant and widely exists in soil environments [4-6]. Because Cd can be easily absorbed by plant roots and transported to the aboveground parts, it not only seriously affects the normal growth of a lot of plants, but also brings harmful effects to human health through the food chain [7-9]. Cd is listed as the first class of carcinogenic substances by the International Agency for Research on Cancer [10].

Cd contamination in soil is of major environmental concern on a world scale, and it is particularly important in China, with the rapid development of industry and agriculture in recent decades [11, 12]. Soil microbes are important parts of the soil environment. The soil microbial community could affect the soil ecosystem structure and functions, and has been suggested as a sensitive indicator to reflect soil quality and predict change of soil ecosystems. Microorganisms respond to excessive heavy metal levels in various ways, including population loss [13], decrease in the functional diversity in the soil ecosystem, and change in the microbial community structure [14, 15]. Cd contamination can disrupt the balance of microbial community structure in soils and reduce the number and biomass of beneficial microbes [16-18]. It has been demonstrated that elevated Cd loadings adversely affect the soil microbial population and their associated activities [19]. The concentration of Cd affects growth, and survival of different microorganisms varies greatly [20]. Cd contamination in agricultural soils not only causes changes in soil microorganisms and their activities, but also results in deterioration of soil fertility [21]. Flue-cured tobacco is one of the important economic crops in China, where the cultivated area and the yield of tobacco leaves ranks first in the world [22]. Being a hyperaccumulator of Cd, flue-cured tobacco can absorb Cd easily from the soil environment [23]. To decrease the potential harmful effects of heavy metals on tobacco as well as to human beings eventually, more attention must be paid to Cd contamination in the tobacco planting soil [24-27]. The objectives of this study are to investigate the effects of different Cd contents on soil bacteria and fungi community structure in flue-cured tobacco planting soil using PCR-DGGE analysis. The result may provide the scientific basis for comprehensive management of tobacco planting soils and remediation strategies for heavy metal contamination in soils.

Materials and Methods

Materials

The tested flue-cured tobacco variety was Zhongyan 100, and soil samples were collected from the Jimo Experimental Station of Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, China. The soil type was Alfisols according to the USDA soil taxonomy classification. The soil samples were air-dried, ground, sieved through a 2-mm sieve, and stored before analyses. Determination of the physical and chemical properties of the soils followed the methods of [28]. Soil pH was measured with a 1:5 soil-to-water ratio. Organic matter was determined by potassium dichromate method. Alkali-hydrolyzable N was determined by alkali solution method. Available P was determined by molybdenum blue colorimetric method after extraction by sodium bicarbonate. Available K was determined by ammonium acetate extraction and flame photometric method. The results were as follows: soil pH: 6.0, the content of organic matter: 11.5 $g \cdot kg^{-1}$, Alkali-hydrolyzable nitrogen: 55.23 $mg \cdot kg^{-1}$, available phosphorus: 27.23 $mg \cdot kg^{-1}$, and the content of available potassium: 87.50 $mg \cdot kg^{-1}$.

Table 1. Cadmium concentrations in different soil treatments of the pot experiment.

Treatment	Application amount of Cd ($mg \cdot pot^{-1}$)	Cd content ($mg \cdot kg^{-1}$)
T1 (CK)	0	0
T2	30	2
T3	60	4
T4	120	8
T5	240	16

Experimental Design

In the soil pot experiment, soil samples (15.0 kg each pot) were placed in plastic pots, which were 40 cm in both height and diameter. A solution containing Cd acetate was spiked into the pot soil to form Cd^{2+} concentrations of 0, 2, 4, 8, and 16 $mg \cdot kg^{-1}$. After culturing for 2 weeks, tobacco seedlings were transplanted in the pots (one for each pot). The tobacco plants were grown in a greenhouse at a temperature range of 20-32ºC. The relative humidity of the soils was kept at approximately 60% by watering with deionized water. The five treatments were designed as follows (Table 1).

Sampling and Analysis

From each pot, the rhizosphere soil subsamples were collected from three tobacco roots at tobacco mature stage. The rhizosphere soil was obtained by gently shaking off the loosely bound soil, while the rhizosphere soil adhering to the root was isolated by more vigorous shaking or by scraping. The three subsamples were pooled into one to form a composite sample. The soil samples were stored in plastic bags in an icebox, and transported to the laboratory, where each soil sample was immediately sieved through a 2 mm mesh and stored at -70ºC for soil microbial community analysis.

Table 2. ICP-MS operating conditions.

Parameter	Value
Forward power	1600 W
Plasma gas flow	15.0 L/min
Carrier gas flow	0.65 L/min
Dilution gas flow	0.55 L/min
Total carrier gas flow	1.20 L/min
Sample uptake rate	1.5 mL/min
QP bias	-98V
Oct bias	-100V
Cell entrance	-125V
Cell exit	-150V
Deflect	-80V

After completing the collection of rhizosphere soil samples, the tobacco plants were divided into three parts: root, stem, and leaf. They were dried at 60ºC, weighed, and then ground into powder.

The powdered samples were digested in HCl:HNO_3:HF (3:3:2) in a microwave oven at 130ºC for 18 min followed by 185ºC for 30 min. After digestion, the solutions were heated at approximately 210ºC to eliminate excess acid. The concentrations of Cd were determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo X Series 2, USA) and operating conditions are present in Table 2 [29, 30].

DNA Extraction

About 0.5 g moist soil from each sample was used for DNA extraction using a Fast DNA SPIN Kit for soil (MP Biomedical, Santa Ana, CA) according to the manufacturer's instructions. The extracted soil DNA was dissolved in 50 ml TE buffer, quantified by spectrophotometer and stored at -20ºC until further use. The bacterial 16S rDNA targeted primer pair consisting of 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3'), and fungal 18S rDNA targeted primer pair consisting of FR1 (5'-AIC CAT TCA ATC GGT AIT-3') and FF390 (5'-CGA TAA CGA ACG AGA CCT-3') both of which were used with a GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') for DGGE analysis of bacterial and fungal communities [31, 32].

PCR-DGGE

A DCode Universal Mutation Detection System (Bio-Rad, Hercules, Calif.) was used for DGGE analysis. Approximately 200 ng PCR amplicons from each sample were electrophoresed on an 8% acrylamide-bisacrylamide gel, with 45-75% denaturant with bacteria, and 40-60% denaturant with fungi at 70V for 15 h in 1×TAE running buffer at 60ºC. The gels were stained for 20 min with SYBR Green I nucleic acid gel stain (1:10,000 dilution) (Invitrogen, Oregon, USA). The gels were visualized and digitalized using a Gel Doc EQ imager (Bio-Rad, USA) combined with Quantity one 4.4.0 (Bio-Rad, Hercules, Calif.). The representative bands were excised, left overnight in 25 µl MilliQ water, reamplified, and run again on the DGGE system to ensure purity and correct mobility of the excised DGGE bands. Correct PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) before cloning.

Cloning, Sequencing, and Phylogenetic Analysis

The purified PCR amplicons of the excised DGGE bands were cloned into a pMD18-T vector (TaKaRa) and transformed into an *Escherichia coli* DH5a competent cell. Six random clones containing correct gene size for each DGGE band were sequenced by the Invitrogen Sequencing Department in Shanghai. DNASTAR software package was used to manually check and compare the clone sequences. One representative clone sequence with high-quality after-sequence comparison from each band was used for phylogenetic analysis. Together with the top three BLAST hits of homologous gene sequences and the gene sequences from cultured and well characterized species in Genbank, the DGGE band sequences were used to build a basic phylogenetic tree by the neighbor-joining method using the software package of MEGA 4.0 (Molecular Evolutionary Genetics Analysis) [33].

Data Analysis

Differences of Cd content and accumulation in the leaf, stem, and root were statistically analyzed for significance using analysis of variance (ANOVA), and treatment means were separated using the least significant differences (LSD) at 0.05 probability level.

Results

Accumulation and Distribution of Cd in Flue-Cured Tobacco

The Cd content in different organs of flue-cured tobacco varied greatly (Table 3), with the Cd content in leaves as the highest, followed by stem and root. The Cd contents in different organs showed a similar increasing trend with the increase of Cd concentration in the pot soil.

Flue-cured tobacco exhibited strong tolerance to Cd contamination; the 16 mg·kg^{-1} Cd in soil had no significant effect on the growth and development of tobacco. The flue-cured tobacco leaves had the strongest accumulation ability to Cd than other organs (Fig. 1), indicating that Cd absorbed from soil was mostly migrated to the leaves. The Cd accumulation in roots was the lowest.

Table 3. Cd content in different organs of flue-cured tobacco.

Treatment	Root (mg·kg^{-1})	Stem (mg·kg^{-1})	Leaf (mg·kg^{-1})
T1 (CK)	0.63^{d}	0.75^{d}	0.65^{d}
T2	1.52^{cd}	2.86^{c}	5.13^{c}
T3	3.39^{c}	4.82^{b}	10.61^{b}
T4	5.54^{b}	5.21^{b}	21.72^{a}
T5	8.40^{a}	8.79^{a}	20.99^{a}

Different small letters in the same column represent significant difference at $P<0.05$.

The Bacteria Community Structure of Rhizosphere Soil

The DNA bands in the PCR-DGGE profiles of rhizosphere bacterial community in different treatment soils showed great differences (Fig. 2). The numbers of the bacteria bands of T2, T4, and T5 treatments were more than those in CK, whereas the bacteria bands in T3 treatment were fewer than CK. In the T3 treatment, some bacteria bands disappeared in the PCR-DGGE profiles of the rhizosphere bacterial community as a response to the exposure to 4 mg·kg^{-1} Cd, and a new band marked No. 4 appeared.

According to the DGGE profiles of the rhizosphere soil in different treatments, 8 dominant bands were sequenced. The bacteria community was divided into 8 clusters according to the phylogenetic tree based on 16SrDNA sequence (Fig. 3), and those clusters were *Sphingobacteriaceae bacterium*, *Candidatus burkholderia*, *Leifsonia leptolyngbya*, *Bacterium ellin*, *Arthrobacter*, *Phycicoccus*, and *Mycobacterium*. The *Bacterium ellin*, *Acidobacteria bacterium Ellen*, and *Mycobacterium* had strong resistance and adaptability to Cd contamination of different concentrations, whereas *Leptolyngbya* had strong adaptability to Cd contamination at a moderate concentration, which could become the dominant population at this level.

The Fungi Community Structure of Rhizosphere Soil

The fungi bands marked as No. 1 and No. 2 appeared as Cd concentration increased. Band 1 especially appeared at the high Cd concentration treatments at 8 and 16 mg·kg^{-1}, while Band 6 disappeared. The fungi bands marked as No. 3 disappeared in the T3 treatment (4 mg·kg^{-1} Cd concentration), but reappeared at higher Cd concentrations. The fungi bands marked as No. 4, No. 5, and No. 7 appeared with different Cd concentrations (Fig. 4).

The fungi community was divided into four clusters according to the phylogenetic tree based on 18SrDNA sequence (Fig. 5), which were *Rhizopus microsporus*, *Phoma herbarum*, *Acremonium sclerotigenum*, and *Fusarium*. The *Fusarium* and unknown species were the dominant fungi species in the fungi community. They existed in all treatments, and had strong adaptability to different Cd concentrations. The *Acremonium sclerotigenum* was sensitive to Cd contamination, and the *Rhizopus microsporus* appeared when Cd concentration was greater than 8 mg·kg^{-1}, suggesting its preference to a high Cd concentration in rhizospheric soil.

Discussion

The significant phytotoxicity of Cd is due to its high transfer coefficient and its relatively weak sorption by the soil colloids, resulting in accumulation in plants [34]. Flue-cured tobacco was able to take up and accumulate Cd. Most Cd absorbed by flue-cured tobacco was accumulated in leaves (accounting for 80% of Cd absorbed), whereas Cd content in both roots and stems was relatively low.

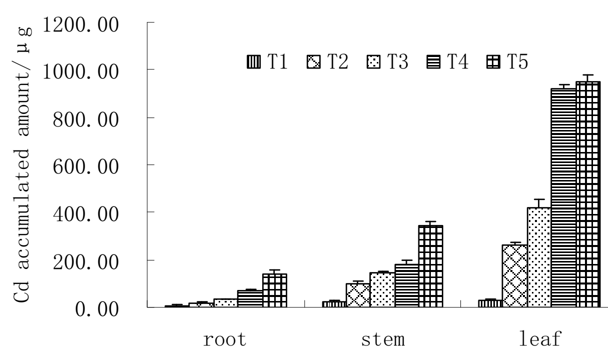

Fig. 1. Cd accumulated amount in different organs of flue-cured tobacco.

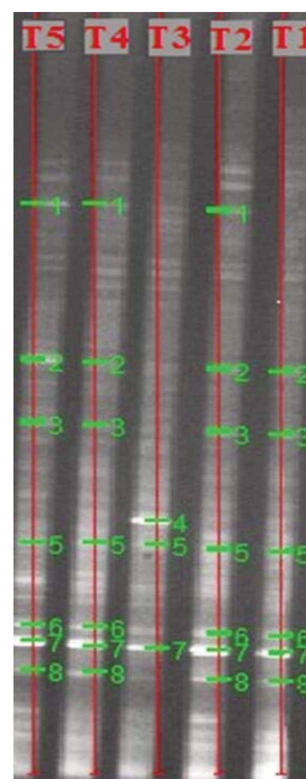

Fig. 2. Effect of different Cd concentrations on bacterial communities in flue-cured tobacco rhizosphere estimated by DGGE profiles.

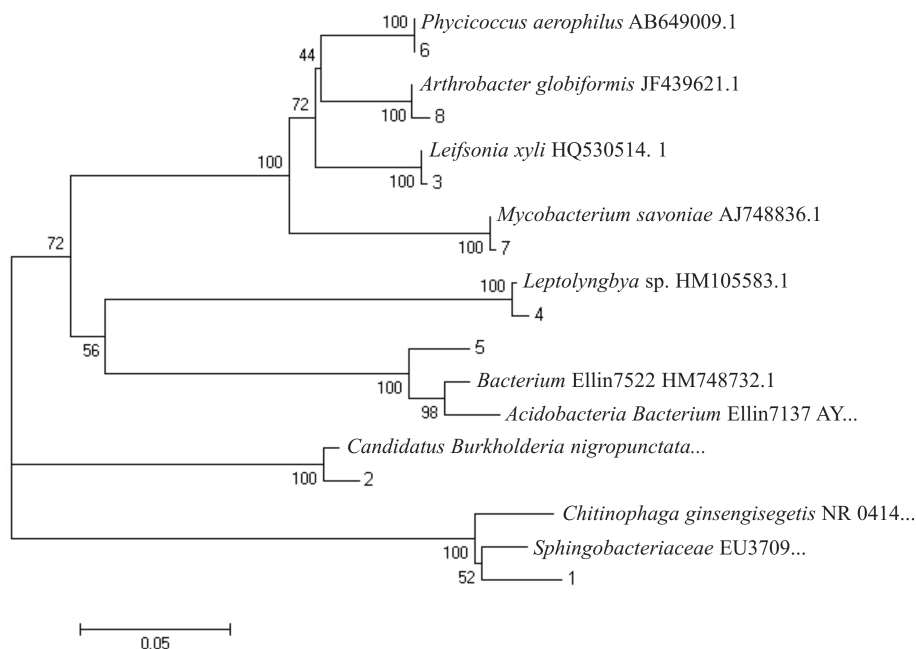

Fig. 3. Phylogenetic tree based on 16SrDNA sequence of bacterial communities in flue-cured tobacco rhizosphere.

These results are consistent with the study by Wu et al. [35]. According to the data provided by Angelova et al. [36], a correlation exists between the total Cd content in the soil and their accumulation in tobacco. The results of our research showed that the Cd content in flue-cured tobacco increased with the increasing concentration of Cd in the pot

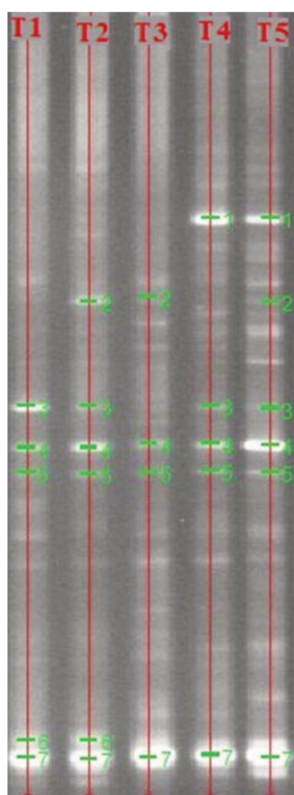

Fig. 4. Effect of different Cd concentrations on fungi communities in flue-cured tobacco rhizosphere estimated by DGGE profiles.

soil. However, the Cd concentration at 16 $mg \cdot kg^{-1}$ had little influence on the growth of the tobacco, suggesting that flue-cured tobacco is highly tolerant to Cd contamination.

The changes of soil microbial community structure can be an indicator of soil nutrient and environmental quality, including soil ecosystem community structure and stability. Previous studies showed that Cd contamination had some effects on the microbial community structure in rhizospheric soil. The response of soil bacteria was influenced by the level of Cd contamination concentrations [37]. In a rice-wheat rotation system, with the increasing concentration of Cd, the bands of PCR product decreased. At a high Cd contamination level, some microbial populations disappeared or declined in numbers [38]. High-level Cd contamination significantly reduced the microbial diversity of different bacterial groups in soils [39], and similar results were found by Hiroki [40] and Ranjard et al. [41]. In this study, the number of microbial bands in the Cd-contaminated soil was reduced for some sensitive microbes, but those resistant microbes could still be separated and identified. Hiroki [39] indicated that the addition of a small amount of Cd into soil caused positive effects on the microbial population. With the increasing concentration of Cd, the resistance of *Pseudomonas* also increased [42]. Other research confirmed that the number of resistant bacteria in Cd-contaminated soil was 15 times more than the normal soil [43]. In this study, we observed that some Cd-resistant microbes appeared with an increase of Cd concentration in flue-cured tobacco rhizosphere, suggesting their strong adaptability to Cd contamination. Compared with the previous results of other soils, the bacteria and fungi bands in flue-cured tobacco rhizospheric soil had no obvious decrease trend at high Cd contamination levels. Therefore, Cd had an important effect on soil microbial community structure with different concentrations in The flue-cured tobacco rhizosphere.

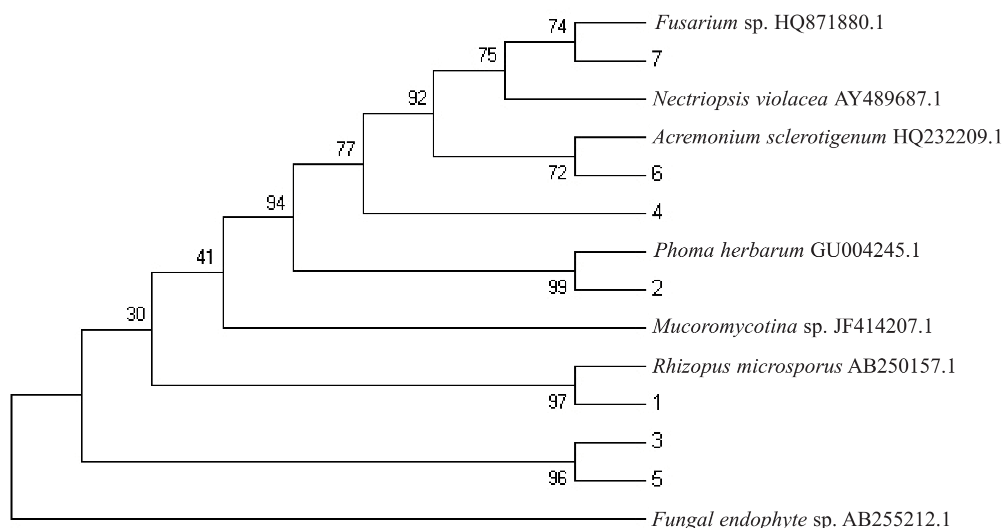

Fig. 5. Phylogenetic tree based on 18SrDNA sequence of fungi communities in flue-cured tobacco rhizosphere.

Conclusions

The Cd accumulation in different flue-cured organs increased with the increasing Cd concentrations in soil. The flue-cured tobacco leaves had stronger accumulation ability to Cd than stems and roots.

Cd contamination had a significant effect on the soil bacteria and fungi community structure of flue-cured rhizosphere. Some specific bacteria and fungi bands appeared or disappeared with the Cd concentration increasing. In terms of bacteria in rhizospheric soils, the *Bacterium ellin*, *Acidobacteria bacterium Ellen*, and *Mycobacterium* had strong resistance and adaptability to Cd contamination of different concentrations. For the fungi, the *Fusarium* and the *Rhizopus* had strong adaptability to higher Cd concentration, but the *Acremonium sclerotigenum* was sensitive to Cd contamination.

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References

1. HU Q., QI H.Y., ZENG J.H. Bacterial diversity in soils around a lead and zinc mine. J. Environ. Sci. **19**, 75, **2007**.
2. ZHANG Y., ZHANG X.L., ZHANG H.W. Responses of soil bacteria to long-term and short-term Cadmium stress as revealed by microbial community analysis. B. Environ. Contam. Tox. **82**, 368, **2009**.
3. SPENCE A., HANSON R.E, GRANT C.N., HOO FUNG L., RATTRAY R. Assessment of the bioavailability of cadmium in Jamaican soils. Environ. Monit. Assess. **186**, (7), 4591, **2014**.
4. ZHAO Z.Q., ZHU Y.G., CAI Y.L. Transport and transformation of cadmium in soil-plant systems and the influence factors. Ecology and Environment. **2**, 283, **2005**.
5. RAN L., LI H.H. Progress in the research of present situation of soil cadmium contamination and its hazards. Journal of Chongqing University of Arts and Sciences (Natural Science Edition). **4**, 70, **2011**.
6. SIX L., SMOLDERS E. Future trends in soil cadmium concentration under current cadmium fluxes to European agricultural soils. Sci. Total Environ. **485**, 321, **2014**.
7. JIANG X.Y., ZHAO K.F. Mechanism of heavy metal in jury and resistance of plants. Chinese Journal of Applied and Environmental Biology. **1**, 94, **2001**.
8. LI X.Z., LI B. Effect of heavy metals on growth, development and quality of plants. Journal of Sichuan Forestry Science and Technology. **4**, 63, **2008**.
9. BAVI K., KHOLDEBARIN B., MORADSHAHI A. Effect of Cadmium on Growth, Protein Content and Peroxidase Activity in Pea Plants. Pakistan J. Bot. **3**, 1468, **2011**.
10. IARC. Beryllium, cadmium, mercury and exposures in the class manufacturing industry. Lyou, Danee: International Agency for Research on Cancer. **58**, 139, **1993**.
11. BAATH E. Effects of heavy metals in soil on microbial processes and populations (a review). Water Air Soil Poll. **47**, 340, **1989**.
12. SHEN G.Q., LU Y.T., ZHOU Q.X. Interaction of polycyclic aromatic hydrocarbons and heavy metals on soil enzyme. Chemosphere. **61**, 1180, **2005**.
13. ZHONG W.H., CAI Z.C. Long-term effects of inorganic fertilizers on microbial biomass and community functional diversity in a paddy soil derived from quaternary red clay. App. Soil Ecol. **36**, 85, **2007**.
14. FROSTEGARD A., TUNLID A., BAATH E. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl. Environ. Microb. **59**, 3607, **1993**.

15. PENNANEN T., FROSTEGARD A., FRITZE H., BAATH E. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. Appl. Environ. Microb. **62**, 421, **1996**.
16. AKMAL M., WANG H.Z., WU J.J., XU J.M., XU D.F. Changes in enzymes activity, substrate utilization pattern and diversity of soil microbial communities under cadmium contamination. J. Environ. Sci. **5**, 803, **2005**.
17. WANG G.H., JIN J., XU M.N., LIU X.B. Effects of plant, soil and soil management on soil microbial community diversity. Chinese Journal of Ecology. **5**, 551, **2006**.
18. ZHANG Y., CUI X.Y., LUO X. Effects of heavy metal contaminations on the ecological functions of soil microbes. Asian Journal of Ecotoxicology. **5**, 36, **2010**.
19. SMITH S.R. Agricultural recycling of sewage sludge and the environment. Wallingford, UK: CAB International. **1996**.
20. BABICH H., STOTZKY G. Sensitivity of various bacteria, including actinomycetes and fungi to cadmium and influence of pH on sensitivity. Appl. Environ. Microb. **33**, 683, **1977**.
21. XIE X.M., LIAO M., MA A.L., ZHANG H.J. Effects of contamination of single and combined cadmium and mercury on the soil microbial community structural diversity and functional diversity. Chin. J. Geochem. **30**, 367, **2011**.
22. ZHANG H.J., HU Z.C., XIE H.X., WANG H.O.,CHEN Y.Q. General situation and development strategy of tobacco production in China. Journal of Anhui Agricultural Sciences. **32**, 14162, **2008**.
23. SU X.K., ZHUANG W.X., LI J.X., SHAO X.H. Impact of heavy metals on tobacco and its control. Chinese Tobacco Science. **4**, 58, **2008**.
24. XU C., GAO M., XIE D.T. Heavy Metal Content Characteristics and Contamination Evaluation in Tobacco Planting Soil of Chongqing. J. Soil Water Conserv. **23**, 142, **2009**.
25. ZOFFOLI H.J., DO AMARAL-SOBRINHO N.M, ZONTA E, LUISI M.V., MARCON G., TOLÓN-BECERRA A. Inputs of heavy metals due to agrochemical use in tobacco fields in Brazil's Southern Region. Environ Monit Assess. **185,** (3), 2423, **2013**.
26. WANG J.J., QIN F.X. Assessment of soil heavy metal content and contamination in flue-cured tobacco planting areas in Zunyi. Guizhou Agricultural Sciences. **10**, 117, **2013**.
27. ORTEGA E., LOZANO F.J., ASENSIO C.M., MONTOYA S., LORENZO M.L. Cadmium distribution in tobacco growing soil fractions: its influence on dried leaf contents. Journal of Environmental Protection. **4**, 2, **2013**.
28. LU R.K. Agricultural Chemistry Analysis of Soil. Beijing: China Agricultural Science and Technology Press. **1999**.
29. LI B., LI D. Determination of trace amounts of cadmium in a plant samples by ICP-MS. Modern Scientific Instruments. **1**, 113, **2011**.
30. GE J.Y., ZHANG J.G. Heavy metal contamination and accumulation in soil and plant species from the Xinqiao Copper Deposit, Anhui Province, China. Anal. Lett. **48**, 544, **2015**.
31. MUYZER G., WAAL E.C., UITTERLINDEN A.G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Appl. Environ. Microb. **59**, 696, **1993**.
32. MAY L.A., SMILEY B., SCHMIDT M.G. Comparative denaturing gradient gelelectrophoresis analysis of fungal communities associated with whole plant cornsilage. Can. J. Microbiol. **47**, 830, **2001**.
33. TAMURA K., DUDLEY J., NEI M., KUMAR S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**, 1597, **2007**.
34. ALLOWAY B.J., AYRES D.C. Chemical principals of environmental pollution (1st ed.). Boca Raton: CRC. **1994**.
35. WU Y.P., YANG H.Q., XU Z.L. Accumulation and distribution of Cd in flue-cured tobacco. Chinese Tobacco Science. **29**, 38, **2008**.
36. ANGELOVA V., IVANOV K., IVANOVA R. Effect of chemical forms of lead, cadmium, and zinc in polluted soils on their uptake by tobacco. J. Plant Nutr. **27**, (5), 756, **2004**.
37. ANYANWU C.U., NWANKWO S.C., MONEK A.N. Soil bacterial response to introduced metal stress. International Journal of Basic and Applied Science. **11**, 110, **2011**.
38. TIAN R., ZHOU H., HUANG J. Effect of elevated atmospheric CO_2 concentration on the soil enzymes and microbial communities under stress of Cd contamination. Journal of Nanjing University (Natural Sciences). **47**, 713, **2011**.
39. ROS M., PASCUAL J. A., MORENO J. L. Evaluation of microbial community activity, abundance and structure in a semiarid soil under cadmium pollution at laboratory level. Water Air Soil Poll. **203**, 230, **2009**.
40. HIROKI M. Effect of heavy metal contamination on soil microbial populations. Soil Sci. Plant Nutr. **38**, 142, **1992**.
41. RANJARD L., RICHAUME A., JOCTEUR-MONROZIER L., NAZARET S. Response of soil bacteria to Hg (II) in relation to soil characteristics and cell location. FEMS Microbiol. Ecol. **24**, 322, **1997**.
42. ROANE T.M., PEPPER I.L. Microbial responses to environmentally toxic. Microbial Ecol. **38**, 359, **1999**.
43. XIANG W. Isolation and characteristics of soil cadmium tolerance and phenanthrene degrading bacteria and study on its influence to diversity of soil microbial communities. Dissertation of master degree of Shanghai Jiao Tong University. **2008**.