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Effects of Soil Sterilization on Growth of *Angelica sinensis* Plant and Soil Microbial Populations in a Continuous Mono-cropping Soil

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Abstract

Angelica sinensis (Oliv.) Diels (family *Apiaceae*) is a perennial herb that has been widely used in Traditional Chinese Medicine. The soil sickness has become one of the major constrains in *A. sinensis* cultivation. A pot experiment was done to evaluate the role of biological nature in *A. sinensis* soil sickness. The pot experiment include three treatments (i) contol, which represent as the soil in pot taken from spring wheat stands, (ii) AA, which represent as the soil in pot taken from *A. sinensis* stands, (iii) S-AA, which represent as the soil in pot was sterilized by steam at 121℃ for 4 h taken from *A. sinensis* stands. Results showed that the plant height, dry weight of aboveground part and roots, root yield and quality, and the activities of SOD and POD in leaves were significantly higher in sterilized replant soil than in non-sterilized replant soil treatment, while the activity of CAT and content of MDA in leaves were declined, which indicated that soil sterilization improved plant haleness and increased the activities of active oxygen scavenging enzymes. The results also demonstrated that soil sterilization can change the number of culturable microbial populations and the species diversity of bacterial functional group. Higher Shannon-Wiener index was found in rhizosphere soils under sterilized soil cropping than that under non-sterilized soil cropping. This suggests that the biological factor played a causal role in the development of *A. sinensis* soil sickness and sterilization of continuous cropping soil could change the composition and structure of soil microbial community, which further promote plant growth and alleviate *A. sinensis* soil sickness. © 2016 Friends Science Publishers

Keywords: *Angelica sinensis*; Soil sickness; Culturable microbial population; Growth parameter; Anti-oxidative enzymes

Introduction

Soil sickness is a reduction in both crop yield and quality, when the same crop or cultivar is grown year after year in the same soil. In general, continuous cropping can affect crop growth and development, decrease yield and quality, and increase disease occurrence (Wu *et al*., 2009; Zhang *et al*., 2010; Zhang *et al*., 2015a). Soil sickness usually occurred in medicinal plant (Yang *et al*., 2014; Zhang *et al*., 2015b). Many researches indicated that there were soil physiochemical property disorders, soil microbial community changes and autotoxicity are responsible for the soil sickness (Yu and Matsui, 1994; Yang *et al*., 2012; Mazzola and Manici, 2012). Previous studies report that soil microflora change is one of the major causes in soil sickness in *Cistus ladanifer* (Hassan *et al*., 1989), peach (Benizri *et al*., 2005), cucumber (Yao *et al*., 2006), *Rehmannia glutinosa* (Zhang *et al*., 2011), Liriope (Zhao *et al*., 2010) and apple (Yim *et al*., 2013).

Angelica sinensis (Oliv.) Diels, is a perennial herb belonging to family *Apiaceae*, and commonly used in Traditional Chinese Medicine since ancient times (Zhang and Cheng, 1989). In addition, *A. sinensis* is widely used as an ingredient in cosmetic and health beverage at present (Chen, 2002; Champakaew *et al*., 2015). In order to meet its demand, areas of continuous cropped for *A. sinensis* have been increased dramatically in the last decade. Because of this, growers face serious problems under continuous cropping including growth retardation, plant mortality and *Ditylenchus destructor* infestation. These reduce not only the root yield but also the quality of *A. sinensis* (Zhang *et al*., 2009). At present, the soil sickness has become one of the major constrains in *A. sinensis* cultivation.

Previous studies on the causative factors for replanting yield and quality decline mainly focused on autotoxicity (Zhang *et al*., 2010) and physiological activity (Zhang *et al*., 2013) in *A. sinensis*. Moreover, the effect of continuous cropping on soil microbial populations in rhizosphere also

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has been investigated (Zhang *et al*., 2015b). However, the possible role of soil microflora in *A. sinensis* continuous cropping obstacle remained obscure.

In this study, effect of soil sterilization on growth of *A. sinensis* plants and soil microflora in a continuous mono-cropping soil was analyzed. The objective was to study the role played by soil microflora in *A. sinensis* continuous cropping problems.

Materials and Methods

Soil

Top loam soil (about 0–30 cm) was collected in October 2008 from the Minxian County (103°34' E, 34°27' N) of Gansu province, China. Twenty samples were taken from *A. sinensis* stands (hereinafter referred to as AA) in which yield declined significantly in *A. sinensis* and from spring wheat (Xihan 1) stands (hereinafter referred to CK) respectively as experimental soil. Three replicates of each soil sample were analyzed for organic matter, pH, total nitrogen (N) content, potassium (K) and phosphorus (P), and available nitrogen (N) and phosphorus (P) concentration. The properties of the AA and CK are shown in Table 1.

The soil samples were screened through a 1mm sieve in order to remove plant residues and stones. A part of AA was sterilized by steam at 121°C for 4 h (Ruan *et al*., 2001), hereinafter referred to as S-AA.

Pot Experiment

The experiment was conducted at the experimental site of the Institute of Radix Angelicae Sinensis in Minxian County, Gansu province in China, during the growing season of 2008. The average temperatures for day and night were 24 and 13°C, respectively and the light and dark periods were 14 and 10 h each day during the whole growing season, respectively.

Ten kg of soil samples was put into plastic pots (30×30×28 cm), respectively the soil samples include three tupes, that is CK, AA and S-AA, respectively. The soil moisture content was raised to 70% of water holding capacity by adding tap water, then the pots were embedded into the soil remaining the up of pot was same height with the ground for eliminating affected by the external conditions using a completely randomized design. *A. sinensis* seedlings were transplanted into each pot on 24 March, and thinned to 4 seedlings/pot at 7 d after they emergence.

Sampling

At 8 May, 23 June and 4 August the stem height was measured to the tip of the youngest visible leaf. At seedling stage (23 June), healthy leaves of *A. sinensis* were collected, and transported to the laboratory in ice-coolers and stored at 4°C until analyzed. The yield and quality was determined at the harvest stage (25 October).

At rootstock thickening (15 August), the soil adhering to the root, designated as 'rhizosphere soil' (Fujii *et al*., 2005), was collected, and the soil samples were mixed, sieved through a 1-mm mesh sieve. The soil samples were transported to the laboratory in ice-coolers and stored at 4°C until analyzed. Care was taken during sampling to prevent cross-contamination of the soils.

Antioxidant Enzyme Activity Determination in Leaves

Antioxidant enzyme extraction and activity determination were carried out following the method of Zhang *et al* (2015b). Generally, each 0.5 g of leaf material was homogenized with extraction buffer containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP and 0.5% (v/v) Triton × 100. The homogenate was centrifuged for 20 min at 12,000 *g* and the supernatant obtained was used for enzyme analysis. All operations were carried out at 0–4°C.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit the photochemical reaction of *NBT* at 560 nm (Zhang *et al*., 2013). One unit of SOD activity was defined as the enzyme amount causing 50% inhibition of *NBT* reduction. SOD activity is expressed as units per mg FM of leaves.

Peroxidase (POD) activity was measured by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation at 25°C (Zhang *et al*., 2013). One unit of POD activity was defined as the increase in absorbance at 470 nm for 1 min due to guaiacol oxidation. POD activity expressed as units per min per g fresh mass (FM).

Catalase (CAT) activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm at 25°C (Zhang *et al*., 2013). One unit of CAT activity was defined as the decrease at 240 nm for 1 min due to H_2O_2 consumption. CAT activity expressed as units per min per g FM. MDA concentrations were measured using the Thiobarbituric acid (TBA) test (Zhang *et al*., 2013).

Enumeration of Culturable Microbial Populations in Rhizosphere Soil

Enumeration of cultivable microbial populations was determined with traditional plate-dilution frequency technique on agar media in Petri plates (Harris and Sommers, 1968). Well mixed 0.1 mL samples of dilutions from 10^{-3} to 10^{-7} (in sterile deionized water) were spread in triplicate onto the following media for cultivable microbe enumerations. Bacteria was determined in the culture medium of Beef-cream and Peptone. Actinomycete was determined in the culture medium of improved Gao 1, and fungi was determined in that of Martin's agar. Azobacter was determined in agar according to Ashby, and aerobic cellulose-decomposing bacteria was determined in agar according to Waksman. Ammonifying bacteria was determined in the culture medium of protein agar medium, and organic phosphorus-solubilizing bacteria was determined in that of Meng

Jina's agar. Inorganic phosphorus-solubilizing bacteria was determined in the culture medium of calcigenol simple and glucose, and kalium-solubilizing bacteria was determined in that of potassium aluminium silicate agar (Zhang *et al*., 2015b).

Analysis of Functional Group Diversity in Rhizosphere Soil

The characteristic parameters including abundance, community diversity and evenness, and dominance concentration were calculated according to the type and amount of functional group (Zhang *et al*., 2015a):

(1) Abundance (P_i) was calculate by Berger-Parker method as $P_i = N_i/N$, where N_i represent the number of individuals in a cluster (or species) divided by the total number of isolates in the sample being analyzed. When $P_i>0.10$ as dominant groups, $0.01<P_i \leq 0.10$ as common groups, $P_i \leq 0.01$ as scarce groups.

(2) Community Diversity (H) was calculate by Shannon-Wiener method as $H = -\sum_{i=1}^{n} Pi \ln Pi$, where P_i represent the proportion of individuals of given species.

(3) Community Evenness (J) was calculate by Pielou method as J = H/lnS, where H represent the diversity of microbe community, S stand for the species number in soil microbe communities.

(4) Dominance Concentration (C) was calculate by Pielou method as $C=\sum Pi^2$, where P_i represent the proportion of individuals of given species.

Statistical Analysis

All experimental data were analysed by ANOVA using SPSS 17.0 software (SPSS Inc., USA) and significant differences were tested using the Least Significant Differences (LSD) test at $P \leq 0.05$. Mean values and standard errors (SE) were presented.

Results

Plant Growth Parameters of *A. sinensis*

Plant height: As shown in Fig. 1A, plant height of *A. sinensis* in continuous cropping treatment was significantly decreased compared to the control at each measured stage. However, effect of soil sterilization on plant height of *A. sinensis* was different with the growth stage. Specifically, the plant height was significantly decreased in continuous cropping by sterilization at the first measured stage, but a significant increase occurred at latter two measured stages.

Plant dry weight: This study indicated that continuous cropping reduced shoot dry weight by 35.42% and root dry weight by 15.70% at rootstock thickening stage (Fig. 1B), as compared to control. However, sterilization treatment increased shoot dry weight by 54.83 % and root dry weight by 13.72%, as compared to continuous cropping.

Yield and quality: This study indicated that continuous cropping decreased the yield, content of essential oils and alcohol-soluble extract by 39.44%, 34.15% and 12.33% (Table 2), as compared to control. However, soil sterilization treatment increased the yield, content of essential oils and alcohol-soluble extract by 43.52%, 31.48% and 7.53%, respectively (Table 2), as compared to continuous cropping, which close to the control.

Antioxidant Enzyme Activity and Lipid Peroxidation in Leaves of *A. sinensis*

Compared with control, continuous cropping was found to significantly decrease the activity of SOD and POD in leaves of *A. sinensis*, but significantly increase the activity of CAT and the content of MDA. However, soil sterilization treatment significantly increased the activity of SOD and POD, decreased the activity of CAT and the content of MDA (Table 3), as compared to continuous cropping. This indicated that soil sterilization could change antioxidant enzyme activity and lipid peroxidation in leaves of *A. sinensis* in a continuous mono-cropping soil.

Enumeration of Culturable Microbial Populations in Rhizosphere soil of *A. sinensis*

As shown in Fig. 2A, the number of culturable fungi of continuous *A. sinensis* cropping soil was significantly greater than that of the control soil at rootstock thickening stage, increased by 22.79% compared to the control. However, the number of culturable actinomycete of continuous *A. sinensis* cropping soil was significantly lower than that of the control soil, decreased by 29.79% compared to the control, and the number of bacteria was not significant difference in continuous *A. sinensis* cropping soil and control.

The number of fungi and actinomycete in sterilized soil was decreased by 52.87% and 81.83%, respectively, compared with those in non-sterilized soil. In addition, bacteria were slightly enhanced (Fig. 2A).

Enumeration of Bacteria Functional Groups in Rhizosphere Soil

This study indicated that continuous *A. sinensis* changed the number of bacteria functional groups in rhizosphere soil of *A. sinensis*. The number of ammonifying bacteria was significantly greater than that of the control, increasing by 96.48% compared to the control (Fig. 2B). Contrarily, the number of aerobic cellulose-decomposing bacteria, organic phosphorus-solubilizing bacteria, inorganic phosphorus-solubilizing bacteria and kalium-solubilizing bacteria in continuous *A. sinensis* cropping soil was decreased by 44.01, 47.19, 71.43 and 21.72%, respectively compared with those in the control soil.

Table 1: The physiochemical properties of soil samples for pot experiment

Soil sample type	Organic matter (%)	Total N content (g kg^{-1})	Total K concentration (g kg^{-1})	Total P concentration (g kg^{-1})	Available N concentration (mg kg^{-1})	Available P concentration (mg kg^{-1})	pH
AA	0.66	0.89	20.74	1.15	29.26	15.84	7.7
CK	0.66	0.96	21.89	1.24	32.45	15.38	7.7

Table 2: Effect of soil sterilization on root yield and quality of *A. sinensis* in a continuous mono-cropping soil

Treatment	Root yield (g·pot^{-1})	Essential oils content (%)	Alcohol-soluble extract (%)
CK	47.71±3.97 [a]	0.82±0.06 [a]	57.45±2.18 [a]
AA	27.04±4.94 [c]	0.55±0.06 [b]	50.35±2.80 [b]
S-AA	37.59±2.56 [b]	0.71±0.08 [a]	54.33±1.57 [ab]

Lines in columns denoted by different letters are significantly at $P < 0.05$ according to least significant difference tests

Table 3: Effect of soil sterilization on antioxidant enzyme activity and lipid peroxidation of *A. sinensis* leave in a continuous mono-cropping soil

Treatment	SOD activity (U·mg^{-1} FM)	POD activity (U·min^{-1}·g^{-1} FM)	CAT activity (U·min^{-1}·g^{-1} FM)	MAD content(μmol·g^{-1} FM)
CK	1.03±0.10 [a]	20.13±1.20 [a]	72.66±5.66 [b]	2.36±0.07 [c]
AA	0.65±0.05 [b]	6.53±0.44 [b]	100.39±9.03 [a]	2.73±0.04 [a]
S-AA	0.96±0.07 [a]	19.58±0.92 [a]	64.81±3.71 [b]	2.57±0.07 [b]

Lines in columns denoted by different letters are significantly at $P < 0.05$ according to least significant difference tests

Table 4: Effect of soil sterilization on parameters of bacterial functional groups diversity in rhizosphere soil of *A. sinensis* in a continuous mono-cropping soil

Treatment	Abundance (P_i)					Total individual No.(N)×10^4	Community diversity(H)	Evenness (J)	Concentration (C)
	AB	ACDB	OPSB	IPSB	KSB				
CK	0.7885	0.1678	0.0092	0.0212	0.0132	13.68	0.6691	0.4157	0.6507
AA	0.9308	0.0564	0.0029	0.0036	0.0062	22.78	0.2981	0.1852	0.8696
S-AA	0.8661	0.1056	0.0070	0.0114	0.0100	10.56	0.4934	0.3066	0.7615

Where AB: Ammonifying bacteria; ACDB: Aerobic cellulose-decomposing bacteria; OPSB: Organic phosphorus-solubilizing bacteria; IPSB: Inorganic phosphorus-solubilizing bacteria; KSB: Kalium-solubilizing bacteria

The number of ammonifying bacteria and kalium-solubilizing bacteria in sterilized soil was significantly decreased by 56.93 and 25.56%, respectively compared with those in non-sterilized soil of continuous *A. sinensis* cropping, however, the number of inorganic phosphorus-solubilizing bacteria was significantly increased by 44.28%, and the number of aerobic cellulose-decomposing bacteria and organic phosphorus-solubilizing bacteria was slightly changed.

Analysis of Functional Group Diversity in Rhizosphere Soil

The abundance of aerobic cellulose-decomposing bacteria, inorganic phosphorus-solubilizing bacteria and kalium-solubilizing bacteria in rhizosphere soil of continuous *A. sinensis* cropping changed compared with the control. Although the total population of functional groups in continuous *A. sinensis* cropping rhizosphere soils was higher than in control soil, H and J were decreased by 55.45% and 55.45%, respectively whereas C was increased by 33.64 % (Table 4).

Abundance of all bacteria functional groups determined, total individual number, H, J and C in sterilized soil of continuous *A. sinensis* cropping changed markedly compared with in non-sterilized soil of continuous *A. sinensis* cropping, which tend to close those in control soil.

Discussion

Soil sterilization could improve growth of *A. sinensis* plants at the later growth stage to greater extent under continuous cropping, and for example, plant height, dry weight, yield and quality of *A. sinensis* plants grown in sterilized soil were significantly higher than those in non-sterilized soil, this is consistent with the results of cucumber (Ruan *et al*., 2001), apple (Leinfelder and Merrin, 2006), pepper (Hou *et al*., 2006) and soybean (Zhang *et al*., 2007). We believe that growth retardation of plant grown in sterilized soil appeared at the early growth stage is due to the fact that microorganisms which played an important role to in nutrient transformation, which were killed by soil sterilization. Soil microbial communities rapidly re-colonize in sterilized soil, and during re-colonization, the community structure changed rapidly with a general trend towards higher diversity and evenness (Marschner and Rumberger, 2004; Yim *et al*., 2013). Soil sterilization eliminated soil-borne pathogens, also increased availability and acquisition of nutrients (Troelstra *et al*., 2001; Costa *et al*., 2006), with the result that plant growth of *A. sinensis* was improved at later growth stage.

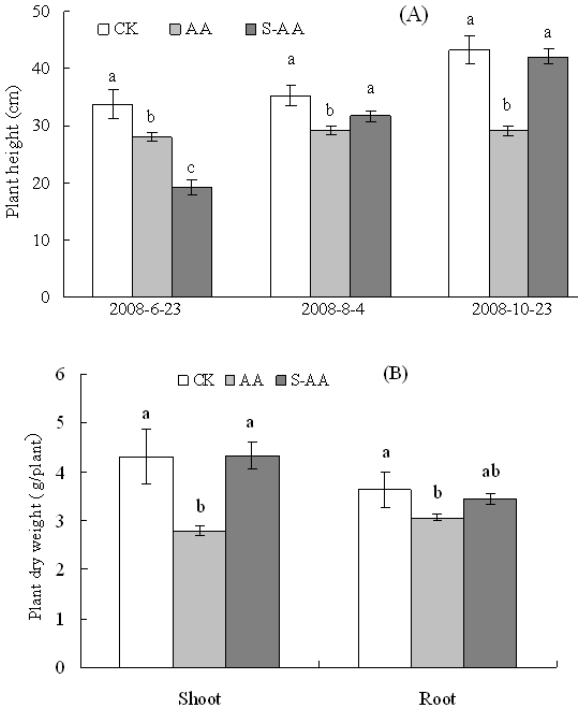

Fig. 1: Effect of soil sterilization on plant height (A) and biomass (dry weight, g) (B) of *A. sinensis* in a continuous mono-cropping soil. Within each growth stage or each plant component, vertical bars (s.e.) with the same letter are not significantly different (P<0.05)

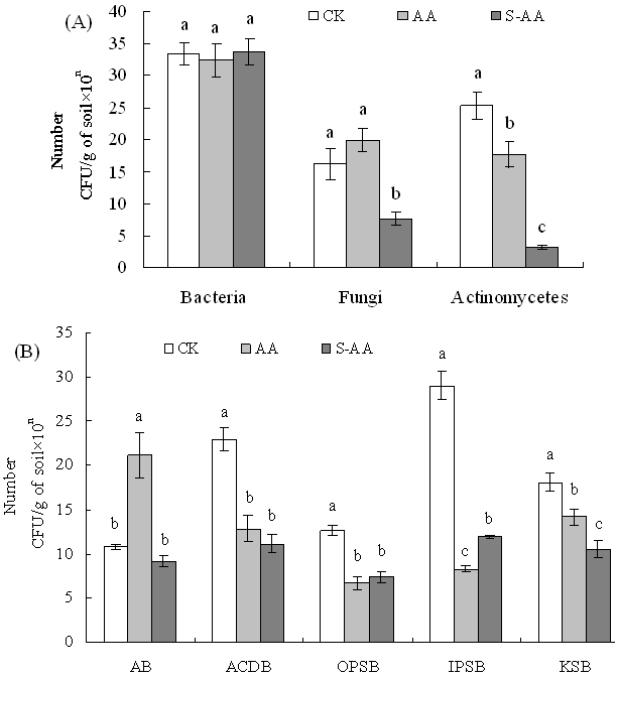

Fig. 2: Effect of soil sterilization on population of microorganisms (A) and populations of bacteria functional groups in rhizosphere soil (B) in a continuous mono-cropping soil. Within each population, vertical bars (s.e.) with the same letter are not significantly different (P<0.05). Where AB: Ammonifying bacteria; ACDB: Aerobic cellulose-decomposing bacteria; OPSB: Organic phosphorus-solubilizing bacteria; IPSB: Inorganic phosphorus-solubilizing bacteria; KSB: Kalium-solubilizing bacteria

Note: Bacteria: CFU/g of soil $\times 10^5$, Fungi. CFU/g of soil $\times 10^2$, Actinomycetes. CFU/g of soil $\times 10^5$. AB, CFU/g of soil $\times 10^4$; ACDB, CFU/g of soil $\times 10^3$; OPSB, CFU/g of soil $\times 10^2$; IPSB, CFU/g of soil $\times 10^2$; KSB, CFU/g of soil $\times 10^2$. Where CFU: Colony forming units

In general, plants generate more ROS and stimulate resistance responses when exposed to stressful conditions (Hancock *et al*., 2002; Thoma *et al*., 2003). Plant possess efficient systems for scavenging ROS that protect them from destructive oxidative reactions (Olmos *et al*., 1994). Anti-oxidative enzymes are the most important components in the scavening system of ROS. The activity of antioxidant enzymes has reported to decrease under continuous cropping of cucumber (Zhang *et al*., 2007) and grape (Guo *et al*., 2010), but increases in pepper (Hou *et al*., 2006).

The effect of sterilized replant soil on the antioxidant enzymes under continuous cropping stress has been reported by Zhang (Zhang *et al*., 2007) who has described increases in activity of SOD, POD, CAT in mono-continuous cropping cucumber leaves and an increase in SOD activity in continuous cropping grape leaves (Guo *et al*., 2010). However, decreases in activity of SOD and POD in continuous cropping pepper leaves and roots (Hou *et al*., 2006). The results of the present experiment showed that the activity of SOD and POD in mono-continuous cropping stressed *A. sinensis* leaves was increased by steam sterilization, however, a decrease in CAT activity was observed. This suggests that in sterilized soil, the stressful condition of continuous cropping was alleviated and making ROS maintain lower levels, so that CAT activities remain a low level. This is consistent with the results of other crops (Lechno *et al*., 1997; Posmyk *et al*., 2005).

Soil microorganisms play an important role in the soil ecosystem, and to a certain extent, soil microbial community composition and changes in types or amounts of soil microorganisms can reflect changes in soil quality (Jiao and Wu, 2004). Microorganisms are also the key to overcome problems associated with continuous cropping and other agricultural practices that detrimentally affect soil health (Liu *et al*., 2010). Further, different diversity indices can reflect better functional of the soil microbial community as affected by soil management. In our study, continuous cropping changes the amount of soil microorganisms and decreases the diversity of bacteria functional groups. This is consistent with the results of Hartmann and Widmer (2006). The result demonstrated that continuous cropping systems have negative effects on soil microbial communities.

Soil sterilization can alleviate the effects of continuous cropping, and improve the ability of plants to adapt to continuous cropping (Zhang *et al*., 2007). The results of our study indicate that soil sterilization can relieve the

detrimental effects of continuous cropping on the quantity and diversity of microorganisms in rhizosphere soil of *A. sinensis* plants, with the result that detrimental effects of continuous cropping was alleviated. This indicated that soil sterilization could as a possible method to eliminate the continuous cropping problem in *A. sinensis* in the controlled experiment condition. However, in the field, for example- soil microbial recovery after sterilization treatments was affected by communities escaped from the treatment, by microbes of deep layers, by those of nearby soils. Therefore, further experiment should to be conducted in the field condition.

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