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Microbial community in *Andrographis paniculata* (*Burm. f.*) Nees in continuous cropping problem

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ABSTRACT

We elucidated the correlations among continuous cropping problems of *Andrographis*, the autotoxins and microbes within its root-zone soil. Soil samples of were collected from different zones of *Andrographis* to assess the 'autotoxic circle' and to analyse the microbial community composition. Seedling growth inhibition by soil and soil extracts decreased with distance from the plant. The microbial constitution in the different *Andrographis* root-zone soils shifted dramatically within 15 cm of the plant characterized by the appearance of various exclusive species. However, beyond this range it was not affected.

Keywords: Allelopathy, *Andrographis paniculata,* autotoxic circle, autotoxicity, continuous cropping problems, microbial community, root-zone, seedling growth, soil extracts.

INTRODUCTION

Andrographis paniculata (Burm. f.) Nees, is important medicinal plant, occurring wild in India and is used both in Ayurevda and Unani system of medicine (2)**.** It is major source of many Chinese medicines (15), hence, cultivated in China. Its demand has increased but its yield and quality has sharply reduced due to continuous monoculture. The adverse effects of continuous cropping problem, restrains the *Andrographis*' sustainable production. Continuous cropping problem, reduces its both yield and quality, increases pests and diseases, exhausts the soil fertility and the allelochemicals accumulates in the soil, which seriously affects the agricultural and medicinal crops production (29). The high demand and low supply of this plant has drawn much attention as cultivation of *Andrographis* plants is hampered by replant failure.

Allelopathy has close relationship with replant failure (19). When allelochemicals from the root exudates or crop residues remains in the soil and accumulates to a certain level, the growth and yield of following crop in continuous cropping is inhibited due to autotoxicity (4,7,11,13,26,31).

Allelochemicals are components of root exudates and they influences the microbial community (1,33). Blum (3) found that the diversity and community structure of microorganisms is related to the type and concentration of phenolic acids in the soil. Qu (24) reported that phenolic acids had significant effects on the biomass, diversity of soil microorganisms, selectively increased the certain microorganisms groups in the soil and

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act as autotoxic substances. However, information on the microbial community distribution within the root-zone soil of *Andrographis* and the influence of *Andrographis* cultivation on microbes is not known. This, investigation aimed to understand the interactions among the plant autotoxins and microbes and to provide a basis to solve the continuous cropping problem in *A. paniculata.*

Figure 1. Test plant- *A. paniculata* (Burm. f.) Nees

MATERIALS AND METHODS

The *A. paniculata* seeds were collected from the Medicinal Botanical Garden, Guangdong Pharmaceutical University, Guangzhou. The soil was sampled from the Guangzhou City (23°20’N, 113°30’E, annual rainfall: 1623-1900 mm, altitude: 18 m, Air pressure: 101KPa, temperature: 24 ℃-32 ℃). This field was never used to grow *Andrographis*, hence, was selected for the container-culture experiment. MS medium was purchased from Beijing Chief-East Tech Co., Ltd (Qwbio). Methanol and n-pentanewere purchased from Guangdong Institute for Food and Drug Control. All chemicals used were of high purity and the solvents used were HPLC spectral grade.

Container-culture experiment

About 3 cm deep surface soil was first removed and then soil up to 20 cms depth was collected. The soil sample was air-dried, ground and passed through 1 mm sieve. One hundred and fifty Kgs of the soil was put in pots (dia 80 cms, depth 40 cms) and in March 2017, one *A.paniculata* seedling (30 days old) was planted in centre of eachpots as per regulations (10) for management. There were five replicates. All pots were irrigated and managed similarly.

After three months（April to June 2017，3-root-zone soil samples **(Fig. 2)** were collected. The surface soil (3 cm deep) was removed before sampling to exclude environmental influences. These soil samples were named as Under: (i). Rhizoshere soil (RS): The soil from 0-5 cm distance from the roots including rhizosphere and rhizoplane soil. (ii). Intermediate soil (IS): The soil within 5 to 15 cms of from the centre of pots and (iii). Distant soil (DS): The soil 15 to 25 cms away from the centre of pots. The soil from the pots that was not used to grow *Andrographis* was collected as control (CK). All the above soil samples were put in an ice box and taken to the laboratory, and stored at −80 ℃ for DNA extraction of soil microbes.

Assessment of 'autotoxic circle' of *Andrographis*

To determine the *Andrographis* 'autotoxic circle' (34), a seedling test was dome to find the toxic effects of various root-zone soils**.** RS, IS, DS and CK soil samples were weighed (20 g) and put into Petri dishes, in triplicate. Twenty *Andrographis* seeds were sown in each petri plate and 10 ml distilled water was added once every 3 days. All dishes were incubated at 27.5 °C under fluorescent light for 12 h (08：00–20：00, fluorescent light intensity 4.68 ± 0.14 × 103 lx.). After 7 days, the radicle length and germination were measured.

Additionally, a radicle test was dome to verify the 'autotoxic circle' using the polar and non-polar extracts from each of these soil samples. For this, soil samples (200 g) were weighed and extracted by mixing with 400 ml HPLC-grade methanol and n-pentane for 24 h, separately. The extracts were then filtered and diluted with corresponding solvents to 400 ml. Eighty ml of each extract was then transferred to glass bottles and evaporated in a draft chamber. Seeds were cultured in the sterilized narrow mouth bottles and managed according to the same conditions as of the seedling tests. The MS formulation was used as the nutrient medium, and there were 5 replications for each soil sample extract. Two hundred ml of MS nutrient medium was added to each repetition of soil sample extract. After 4 weeks, the roots of plantlets were washed and scanned using an Epson Expression 10000XL scanner (Epson Co., Ltd., Long Beach, CA, USA). Root length was determined by analysing the scanned images using WinRHIZO version Pro2007d software (Regent Instruments Inc., Quebec, Canada)**.** The extent of inhibition (% I) was determined by comparing the radicle length in the soil and the plantlet root length grown with extracts and control.

$$\% \text{ I}=\frac{\text{control}-\text{treatment}}{\text{control}}\times 100\ \%$$

Where, I >0: Growth inhibition, I <0: Growth promotion (16).

Microbial community in different root-zone soils

DNA was extracted from soil (0.25 g) by the Power Soil DNA Isolation kit (MOBIO Laboratories, Inc., US). The quality of extracted DNA was checked by Gold View staining after 1 % agarose gel electrophoresis (AGE). DNA was stored at -20 ℃ until subsequent analysis. To assess the bacterial communities, we amplified the V4–V5 hyper-variable regions of bacterial 16S ribosomal RNAs (rRNAs; *Escherichia coli* positions 515–907) using the primer set (*5): F515: 5′-GTGCCAGCMGCCGCGG-3′, and primer R907* **(22):** *5′-CCGTCAATTCMTTTRAGTTT-3′.* To assess bacterial communities, ITS1F/ITS2R of fungus ITS rRNA gene using primer set: ITS1F：5′-CTTGGTCATTTAGAGGAAGTAA-3′, and primer ITS2R：5′-GCTGCGTTCTTCATCGATGC-3′. PCR amplification was done with 25-ml 2 × premix (TaKaRa), 0.5 ml 20 mM each forward and reverse primer and 50 ng of DNA, and the volume was made to 50 ml with double-distilled water. Each sample was amplified in triplicate with the 50 ml reaction under the following conditions: 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 30 s; with a final extension at 72 ℃ for 10 min **(8).** PCR products from each sample were pooled together, purified with an agarose gel DNA purification kit (Agarose Gel DNA Purification Kit，TaKaRa). The purified product was mixed in equal amounts and sequenced using Illumina Miseq and the sequencing results were analyzed using QIIME software (Quantitative Insights Into Microbial Ecology, Version 1.8.0). The diversity index of a sample was evaluated using Mothur (version v.1.30) software. To compare the diversity index between samples, the number of sequences contained in the sample was normalized when analysis. The diversity index of each sample was counted at the 97% similarity level **(30).**

STATISTICAL ANALYSES

Excel 2010 and SPSS 17.0 software was used for statistical significance of all basic data. Each value was expressed as the mean of three replicates ± SE.

RESULTS AND DISCUSSION

Autotoxic circle of *Andrographis*

The radicle length of *Andrographis* seedlings in RS and IS soils was significantly inhibited, while the radicle length in DS was similar to control (**Figs. 2 and 3).** The strongest inhibitory effect was observed in RS soil (% I = 26.1 %) and less in IS soil (% I = 10.4 %). These results indicated that the autotoxic effects of *Andrographis* were strong within 15 cm from the plant and decreased with distance.

To exclude the influence of microbes on the assessment of ‘autotoxic circle’, the polar and non-polar chemicals within different root-zone soils were extracted and their combined effects were evaluated on the root growth of plantlets. The results were similar in seedling tests with the soil samples**.** The strongest inhibitory effect was observed with the RS extract (%I = 59.6 %) followed by IS extracts (%I = 26.3 %) and the inhibition effect of DS extract was the weakest (**Fig. 4**). These similar results indicate that the different compounds within the different root-zone soils were responsible for the formation of *Andrographis* ‘autotoxic circle’ and its maximum diameter was about 20 cm from the main roots.

Microbial community in different root-zone soils

High-throughput sequencing revealed the diversity of microbial communities in different samples at the species level (**Figs. 5 and 6**). The relative abundances of 10 bacteria (each relative abundance, >1 %) are shown in **Fig. 5**. The 10 bacteria were detected in all samples; however, their relative abundances varied in different samples. The abundance of *uncultured_bacterium* was 12.2 % in RS, the dominant bacterium. However, the dominant bacteria in IS and DS in uncultured bacterium_*f_ Gemmatimonadaceae* and uncultured bacterium_*f_v Nitrosom-onadaceae*, were 24.6 % and 35.65 %, respectively.

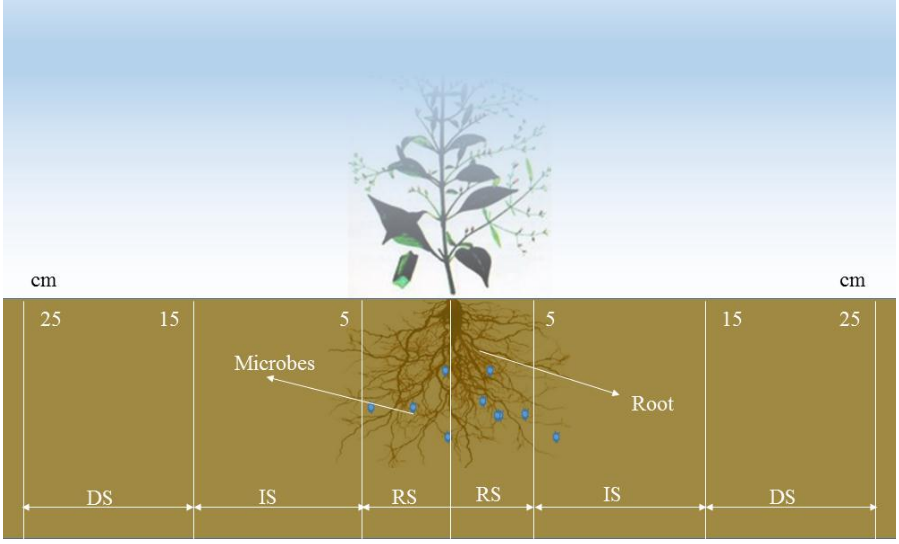

Figure 2. Different root-zone soils of *Andrographis*

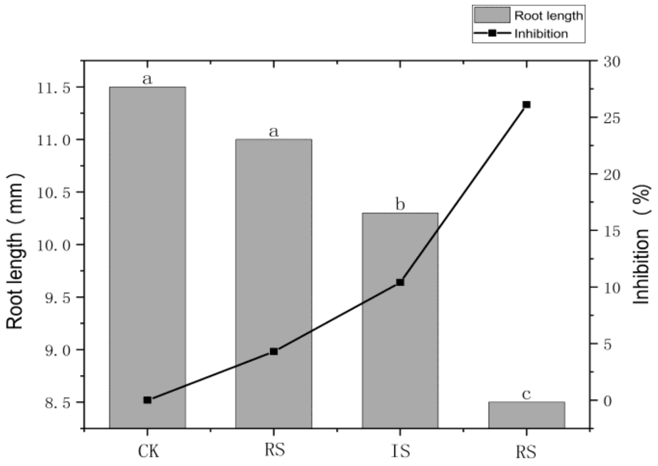

Figure 3. Inhibition of radicle growth of *Andrographis* seedlings by different root-zone soils
Different letters indicate significant differences among different radicle length at $P < 0.05$.
RS : Soil within 5 cm ; IS : Soil within 5 to 15 cm, DS : Soil within 15 to 25 cm from the plant.

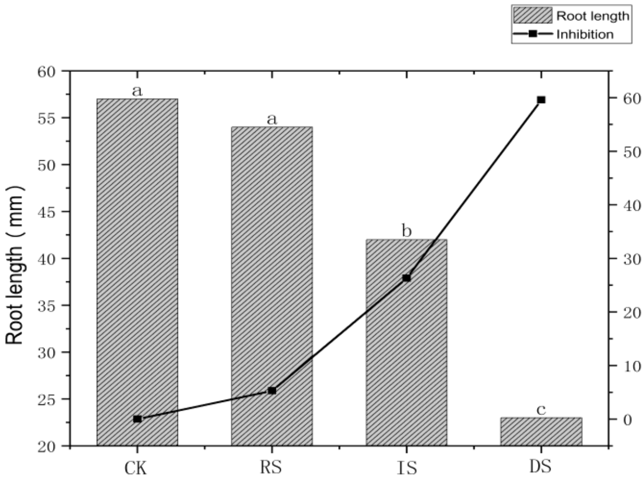

Figure 4. Inhibition of radicle growth of *Andrographis* by different root-zone soil extracts
Different letters indicate significant differences among different radicle length at P < 0.05. For other details in **Fig. 3**.

The relative abundances of top 10 Fungi (each relative abundance, >1 %) are shown in **Fig. 6**. Ten fungi were detected in all samples**;** however, their relative abundances varied in samples. The abundance of *Polyschema_sclerotigenum* was 6.3 % in RS and was the dominant fungus. However, the dominant fungi in IS and DS are *Humicola phialophoroides* and *Pisolithus orientalis*, accounting for 3.4 % and 24.8 %, respectively. Additionally, the Ribosomal Database Project (RDP) classifier was used for the taxonomic analysis at 97 % similarity level of OTU representative sequences. The diversity indices mainly include: richness index Chao 1, diversity index Shannon, and sequencing depth and Coverage **(Table 1).**

Table 1. Soil microbial diversity and richness of different root-zone soils

Root zone soils	Bacterial community				Fungal community			
	OUT	Chao 1	Shannon	Coverage	OUT	Chao 1	Shannon	Coverage
RS	3938	4730a	7.22a	0.9985	1231	1170a	5.31a	0.9982
IS	2177	2818b	6.23ab	0.9969	792	831ab	4.08b	0.9990
DS	1892	2405b	5.95b	0.9911	481	492b	4.02b	0.9998

Different letters represent significant difference (P < 0.05).
RS : Soil within 5 cm ; IS : Soil within 5 to 15 cm, DS : Soil within 15 to 25 cm from the plant.

More than 99 % of coverage indices indicated that the sequencing results at similarity level of 0.03 can reflect the true microbial diversity in tested samples. Overall, the microbial diversity and evenness index of the three soil types exhibited a descending order: **RS > IS > DS.** In the rhizosphere soil, the bacterial diversity and the fungal diversity indices were significantly higher than that in the intermediate and distant soils. These findings indicate that the microbial community in the soil changed due to *Andrographis* cultivation and the microbial constitution in the different *Andrographis* root-zone soils shifted dramatically, particularly within 15 cm of the plant roots was that characterized by the appearance of various exclusive species. However, microbes in the soil beyond this range were slightly affected.

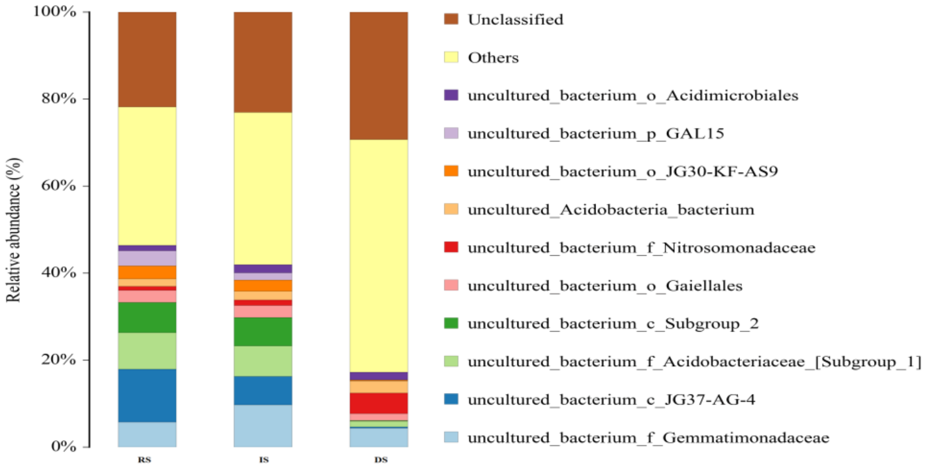

Figure 5. Relative abundances of different bacteria in different root-zone soils
RS : Soil within 5 cm ; IS : Soil within 5 to 15 cm, DS : Soil within 15 to 25 cm from the plant.

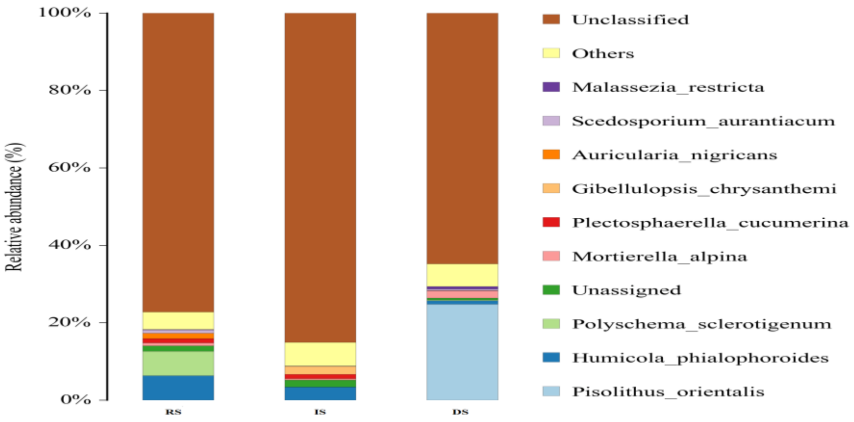

Figure 6. Relative abundances of Fungus in different root-zone soils
RS : Soil within 5 cm ; IS : Soil within 5 to 15 cm, DS : Soil within 15 to 25 cm from the plant.

Various allelochemicals/autotoxins are involved in the plants' allelopathy/autotoxicity and the autotoxicity is regarded as one of the important factors in continuous cropping problems (21,28,37). Our results demonstrated that the soils cultivated with *Andrographis* develop an autotoxicity effect on the germination of *Andrographis* seeds, and this effect decreases as the distance from the plant increases.

Assessment of autotoxic effect under aseptic conditions showed similar results with soil culture conditions, implying that an 'autotoxic circle' was formed during the *Andrographis* cultivation.

Tracking the potential functions of the identified microbes is difficult as most of them were unknown. However, some species that we identified belong to the same genera which are confirmed to have positive effects on plant growth. For instance, some species of *Gemmatimonadaceae* and *Humicola* promotes plant growth due to their antagonistic activity, the production of IAA or specific substance to activate resistance to diseases (9,14,32). Some species of *Actinobacteria* improves the plant nutrition and establishes relationships with other soil fungi (23). *Acidobacteria* degrades the soil polysaccharides and consequently, play a role in the C cycle (18). On the other hand, some species of these genera have negative effects on plant growth due to their broad-spectrum pathogenic activity (6,12,25,35). Zhang *et al* (36) reported that continuous cropping of *R. glutinosa* decreased the bacterial species and simplified the bacterial community structure (36). Thus, the changes in the microbial structure of the soil zones may be one important reason in plant continuous cropping problems.

During the development of continuous cropping problems, some specific microbes may play an important role to catalyse, transform and degrade the autotoxins. In our studies, although we see a change in the microbial spectrum, but it is not possible to assess the microbes role precisely. Thus, more studies are necessary not only on the various soil microbes, the autotoxins and the plants' abnormal metabolism but also on their interactions and functions in mediating continuous cropping problems.

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