

Autotoxicity in the cultivated medicinal herb *Andrographis paniculata*

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ABSTRACT

To determine the autotoxicity in *Andrographis paniculata*, we prepared the aqueous extracts of leaves, stems and replanted soil to determine their autotoxicity. All extracts inhibited the seed germination and seedling growth at higher concentration. Using the HPLC, the Andrographolide, ferulic acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid were detected in *A. paniculata* cultivated soils but not in uncultivated soils. In bioassay, the 5-phenolic compounds (ferulic acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid) showed toxicity to seed germination and seedling growth of *A. paniculata*. Adding activated carbon to the growth substrate in pots significantly improved the growth of *A. paniculata* and enhanced the biomass and chlorophyll content.

Key Words: Allelochemicals, *Andrographis paniculata*, autotoxicity, HPLC, phenolic compounds, replanting problem, seed germination, seedling growth.

INTRODUCTION

Autotoxicity is intraspecific allelopathy that exists in natural, semi-natural and agricultural plant communities leading to population deterioration, regeneration failure and subsequent yield decline (33). Replant failure of crops, is reported for more than two centuries, and decline in plant productivity has been attributed to toxic agents released by the decomposing plant tissues (32), nutritional disturbances (15) and diseases (9). Autotoxicity is major factor in continuous cropping leading to reduced seed germination and poor growth (6). The autotoxicity plays an important role in the replant failure of many commercial crops in agriculture and forestry (7,16,19,20,24-26,28,31,39,42). Compared with common crops, medicinal plants are reported to be more susceptible to autotoxicity. In China, land for cultivation of medicinal plants is limited, hence, continuous cropping is a practised. Severe autotoxicity is reported in many Chinese medicinal plants : *Panax quinquefolium* (45), *Panax Notoginseng* (37), *Rehmannia glutinosa* (8), *Codonopsis pilosula* (44), *Scutellaria baicalensis* (43) and *Glycyrrhiza uralensis* (35).

Andrographis paniculata is an important annual medicinal herb in Southern China. It is cultivated in a continuous cropping, hence suffers from replanting problem. Autotoxicity is presumed to be one of the reasons. Its leaves and roots are used for medicinal purposes. It is commercially grown in Southern China and has become an important economic commodity for pharmaceutical production. However, one of the major

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problems is that replanting usually causes decline in its yield and quality. *A. paniculata* is rich in terpenoids, flavonoids and polyphenols and these compounds are phytotoxic (36).

This study aimed to examine the autotoxicity as a functional process in *A. paniculata* replanting. We designed laboratory and greenhouse experiments to identify some of the chemicals involved in causing autotoxicity and studied their effects on seed germination and seedling growth.

METHODS AND MATERIALS

Materials

Seeds of *A. paniculata* were obtained from Guangzhou University of Chinese Medicine, Guangzhou, China. Soil samples were collected from the Medicinal Plants Garden, Southern Medical University, Guangzhou, China (N 23°19', E 113°34', Height above sea level 26 m). Subtropical monsoon climate, average annual rainfall : 1694 mm, relative humidity : 68%, and a mean annual temperature : 21.8°C (4). The soil samples were collected from the plough layer of cultivated fields at 0-15 cm depth, where *A. paniculata* had been grown for double harvests in September, 2008. The control soil samples were taken from the uncultivated soil at 0-15 cm depth.

The standard compounds used for HPLC analysis were andrographolide, ferulic acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid (Sigma-aldrich, St Louis, MO, USA). The solvents were HPLC grade. All solvents and distilled water prepared for HPLC were filtered through 0.22- μ m milipore membrane. Activated carbon powder and HCl were purchased from Beijing Chemical Reagent (Beijing, China). HCl was of analytical reagent grade.

Bioassay

Plants of *A. paniculata* were grown in round 2-L plastic pots (dia 15 cm, depth 12 cm, three seeds per pot) containing 0.5-mm sands in a greenhouse at South China Agricultural University, Guangzhou, China on March 1, 2018. The plants were fertilized twice a week with Hoagland nutrient solution (14). After two months (May 1, 2009), plants were harvested using fresh leaves and stems (10 g) were separated, cut into 2-cms segments and soaked in 100 mL distilled water for 48 h at room temperature (20-24°C). The extracts were filtered twice through two layers of filter paper (No.1, 10 cm dia) and further diluted with distilled water to get concentrations of 4, 10 and 20 mg dry weight/mL for leaf leachates and 7, 17.5 and 35 mg dry weight/mL for stem leachates. Plant moisture was determined by oven drying (70°C for 3 d).

To prepare soil extracts, the replanted soil was air-dried in shade under natural conditions in laboratory for 2 weeks. Soil samples (200 g) were mixed well, ground to pass 1 mm sieve and soaked in 100 mL distilled water for 48 h at room temperature. The soil extracts were filtered twice through filter paper and diluted with distilled water to concentrations of 0.1, 0.25, and 0.5 g/mL. The pH of the extracts was adjusted to 7.0 with 1 M HCl or NaOH.

To determine the effects of plant and soil extracts on seeds germination, seeds of *A. paniculata* were surface sterilized with 5 % NaClO for 10 min and then washed thrice with sterile distilled water. Fifty seeds were sown in sterilised 9 cm dia petri plates lined with 2 layers of filter paper containing 5 mL of aqueous extracts as per treatments. Distilled water was used as control. Petri dishes were sealed with parafilm to prevent water loss and

incubated in growth chamber [30 ± 1 °C with 14 h light and 10 h dark]. After 12 days, the number of germinated seeds were recorded and the seedlings root length and shoot length were measured with scale. All experiments were done in triplicate.

Effects of Activated carbon on *A. paniculata* seedlings growth

To determine if adsorption of toxic chemicals on carbon would improve the seedling growth, 20 plastic containers (22 cms \times 14 cms \times 7 cms) were filled with 1.5 kg sand sieved through 1 mm sieve. They were divided into two groups. In one group 20 g activated carbon was mixed with the sand in each pot (ratio of 20:1500). The other group without activated carbon served as control. Six newly sprouted seedlings (35 days) were transplanted per pot and fertilized with the Hoagland nutrient solution every once in 5-days. Pots were randomly rearranged three times per week in greenhouse. After 90 days (September 6, 2009), the plants were harvested, the above ground and underground parts were separated, dried at 70°C for 3 d and weighed.

Before drying, the leaves Chlorophyll a and b content were determined spectrophotometrically (UV-2450, Shimadzu, Japan) in 95% ethanol leaf extracts as per method of Knudson *et al.* (21).

HPLC Quantification of Autotoxins in monoculture soil

(i). Determination of Andrographolide: Twenty g of air dried monoculture soil was finely ground, placed in 250 mL flask containing 100 mL of 85 % ethanol and sonicated by ultrasonic vibration at room temperature. The extracts were filtered through filter paper (20 μ m) and the filtrates were dried using rotary evaporator at 40 °C. The dried material was dissolved in 10 mL HPLC grade methanol. Andrographolide in the extract was identified by HPLC Agilent 1100 system (Palo Alto, CA, USA), fitted with a Hypersil ODS C18 column (4.0 mm \times 250 mm), and an auto-injector with a 25- μ l sample loop. The mobile phase was water and methanol (1:1), at a flow rate of 1 mL/min and detection wavelength was 225 nm. Retention times for the standard and the major peak in the extract were recorded.

(ii). Analysis of phenolic acids: Twenty-five g air dried monoculture soils was added to 25 mL of 1 mol NaOH in a 100 mL flask and agitated for 24 h on a reciprocal shaker. The soil suspension was then centrifuged at 5,000 g for 15 min and the supernatant was filtered through two layers of filter paper (No.1. 10 cms dia). The pH of filtrate was adjusted to 2.5 with HCl and incubated for 2 h at the room temperature and then centrifuged at 8000 xg for 10 min. The suspension was kept at 4°C (12). Each soil sample was processed in duplicate and the samples were analyzed separately using HPLC with the detection wavelength of 280 nm as described above. The mobile phase was 25% methanol and 75% glacial acetic acid (pH 2.7). Identification and quantification of the phenolic acids in the soil extracts was done from their retention times and peak heights compared with the retention time of standards. Concentration of phenolic acids is expressed in μ g.g⁻¹ soil.

Bioassay for Autotoxins

Seeds of *A. paniculata* were surface-sterilized with 5 % NaClO for 10 min and then washed thrice with sterile distilled water. The standard compounds including andrographolide and five phenolic acids (Ferulic acid, vanillic acid, caffeic acid, syringic

acid, *p*-hydroxybenzoic acid) were diluted with methanol to get different concentrations (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 mg/mL for andrographolide and 0, 5, 10, 20 µg/mL concentrations). Five mL samples of test concentration were transferred in sterilised 9 cm dia petri plates lined with 2 layers of filter paper. The solvents were evaporated in draft chamber for 2 h. Fifty seeds were separately placed on the filter paper and moistened with 5 mL distilled water. Distilled water was used as control. The Petri dishes were covered and wrapped with parafilm to prevent water loss and placed in growth chamber [30°C with 14 h light and 10 h dark]. One mL distilled water was added to each Petri dish every once in two days to maintain moisture. Each concentration and control were replicated thrice. Germination (%), root and shoot length of seedlings were measured on day 12 after treatment.

Statistical Analysis

All tests were done in triplicate and data are expressed as mean \pm SD and analyzed by SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA). Differences in toxic effects were analyzed using One-way ANOVA followed by Duncan test at 0.05 significance level. T-test was used to analyze differences of biomass, chlorophyll and the content of autotoxin in the soils.

RESULTS AND DISCUSSION

I. Aqueous extracts

The aqueous extracts of fresh leaves and stems significantly inhibited the seed germination and root growth in all test concentrations (Fig. 1a & 1b). The degree of inhibition increased with increasing concentrations i.e. concentration dependent. The bioassay of plant extracts showed that chemicals toxic to the plant are present in *A. paniculata*. To establish our observations, aqueous extract of soils was used to determine the potential impact of the root exudates released into soil environment (2). The soil aqueous extracts at low concentrations (0.1, 0.25 g/mL) did not inhibit germination except at 0.5 g/mL. However, compared with control, soil aqueous extracts of all concentrations significantly inhibited the root growth (Fig. 1a & 1b). No significant inhibitory effects of plants and soil extracts were observed on shoot growth ($P > 0.05$) (Fig. 1c).

II. Activated carbon

Since it was found that both tissue and soil extracts affected seed germination and seedling growth as described above. To further verify the autotoxic effect of root exudates, activated carbon was added to the in sand culture. The activated carbon, which adsorbs the organic chemicals and widely used in ecological studies was used to clarify the role of allelopathic substances (18, 27). Addition of activated carbon to the growth medium significantly increased the above ground and underground biomass of *A. paniculata* plants than control ($P < 0.05$) (Fig. 2a). Chlorophyll a and b were also enhanced ($P < 0.05$) (Fig. 2b). Addition of activated carbon significantly improved *A. paniculata* growth, leading to an increase in plant biomass and chlorophyll contents. This suggested that the better growth of plants was perhaps due to the adsorption of allelochemicals released by the plant roots by the activated carbon.

The physiological mechanisms of plant autotoxicity are complex, hence, not many investigations reported. Ferulic acid found as an autotoxin in rice plants, inhibits the rice

root elongation by modulating signalling and biosynthesis of ethylene and jasmonic acid (5). Further studies are needed to understand the mechanisms of replant failure in *A. paniculata* cultivation and the mechanism of action of the phenolics.

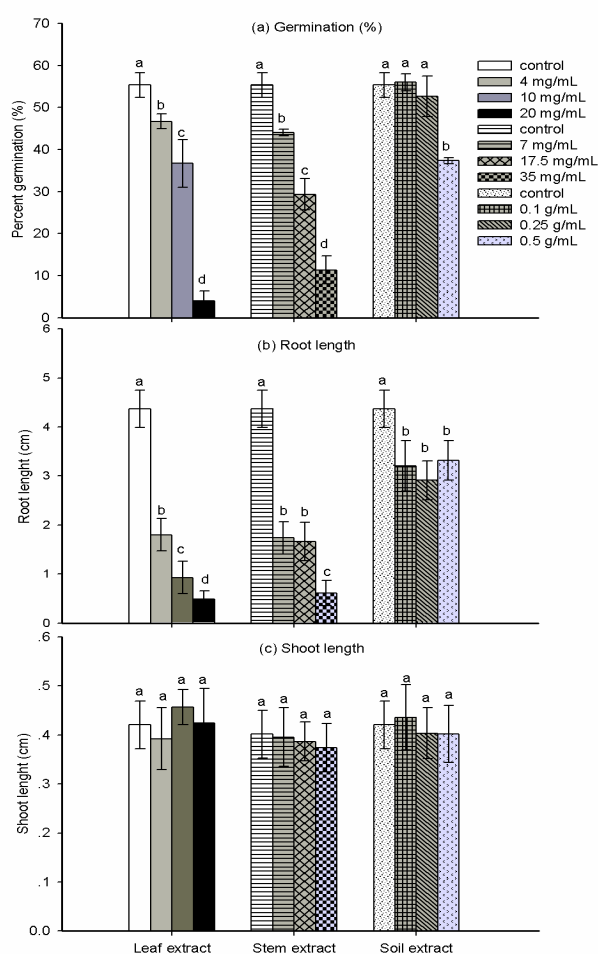


Figure 1. Effects of plant and soil aqueous extracts concentrations on seedling growth of *A. paniculata*.

Different letters above bars indicate significant differences among treatments ($P < 0.05$) according to Duncan's multiple range tests.

III. Quantification of autotoxins in monoculture soils

To determine whether the autotoxic compounds existed in *A. paniculata*-cultivated soils, the phenolic acids were extracted from *A. paniculata*-cultivated and non-cultivated soils and the extracts were analyzed with HPLC (Fig. 3). Five phenolic compounds (Ferulic acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid) and andrographolide were detected in 2-years old monocultured soil, while the uncultivated

soil had only three phenolic compounds (vanillic acid, syringic acid and *p*-hydroxybenzoic acid). Caffeic acid, ferulic acid and andrographolide were not found in uncultivated soil. The concentration of these chemicals differed in cultivated and uncultivated soils. This indicated that five phenolic compounds and andrographolide were found in abundance in *A. paniculata*-cultivated soils, whereas, caffeic acid, ferulic acid and andrographolide are either absent or present (at much lower concentration) in non-cultivated soils (Fig. 3). Plant phenolic compounds are the major allelochemicals implicated in allelopathy and autotoxicity (23,29,30). Besides, the andrographolide is major medicinal constituent in *A. paniculata*. We found that the contents of five major phenolic compounds and andrographolide were higher in *A. paniculata*-cultivated soil.

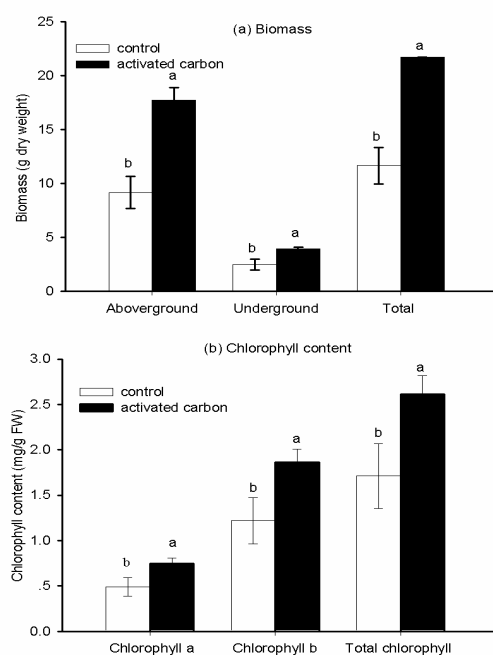


Figure 2. Effects of activated carbon on biomass and chlorophyll content of *A. paniculata*. Different letters above bars indicate significant differences between treatments ($P < 0.05$) according to T- tests.

The role of phenolic acids as allelochemicals has been reviewed (17). In *Panax quinquefolium* L., (13), 5- phenolic acids (Vanillic acid, *p*-coumaric acid, azelaic acid, palmitic acid and (9Z,12Z)-linoleic acid) identified from its fibrous roots significantly reduced the growth of their seedlings. By using the bioassay-guided isolation technique, 8 common phenolics (Coumarin, vanillin, isovanillin, *p*-hydroxybenzoic, vanillic, benzoic, cinnamic, and ferulic acids) and a novel cyclic dipeptide were isolated from a 20-year-old sick soil of replanted Chinese fir (22). Benzoic, salicylic, malonic acids and *p*-hydroxyphenylacetic acids have been reported as autotoxins in three beans (*Pisum sativum*, *Phaseolus vulgaris* and *Vicia faba*) (1). Hence, the presence and accumulation of

many phenolic acids in the soil may be responsible for the plants autotoxicity.

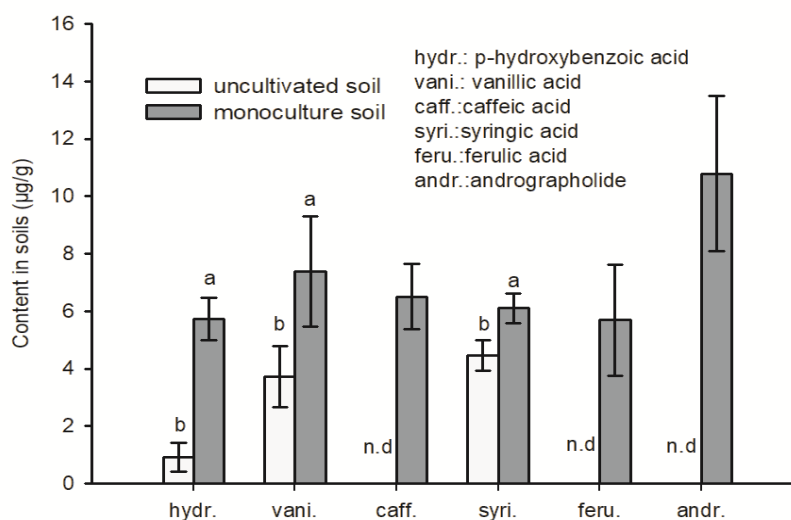


Figure 3. Phenolic acids and andrographolide contents in monoculture and uncultivated soil. Different letters above bars indicate significant differences between treatments ($P < 0.05$) according to T- tests. 'n.d.' indicates that corresponding substance was not detected.

IV. Autotoxicity compounds in monoculture soils

Phytotoxicity of five phenolic acids and andrographolide found in *A. paniculata*-cultivated soils was evaluated (Fig. 4). The five phenolic acids inhibited the seed germination at 5-20 µg/mL concentrations. The effects of these compounds on germination were not dose-dependent, and differences were not significant among different concentrations (Fig. 4a). The root growth of *A. paniculata* was more sensitive to these compounds than shoot. As a result, the root length was significantly reduced. The effect on root growth was dose-dependent and thus higher the concentration, the greater was the inhibition i.e. dose dependent (Figs. 4b, c). Andrographolide showed no significant inhibition on germination ($P > 0.05$), but significantly reduced the root length. Reduction in root length was dose-dependent > 0.05 mg/mL (Table 1). Bioassays demonstrated that these compounds show an autotoxic effect on the growth of *A. paniculata* seedlings. In these studies, a dose-dependent effect of phenolic acids on seed germination was not seen. One possibility is that the lowest concentration tested were too high. Hence, further studies at lower concentrations are required to verify this presumption.

Our results partially explain the *A. paniculata* replant failure. Although the tissue extracts and soil extracts inhibited the seed germination and plant growth, but it is difficult to quantify their effects. Addition of activated carbon overcomes the growth inhibition, but whether due to the adsorption of only phenolics and alkaloid, needs further studies. Further studies are also needed to develop more sustainable practices to maintain the agronomic productivity and economic sustainability in medicinal plants (3,10,11,34,38,40,41).

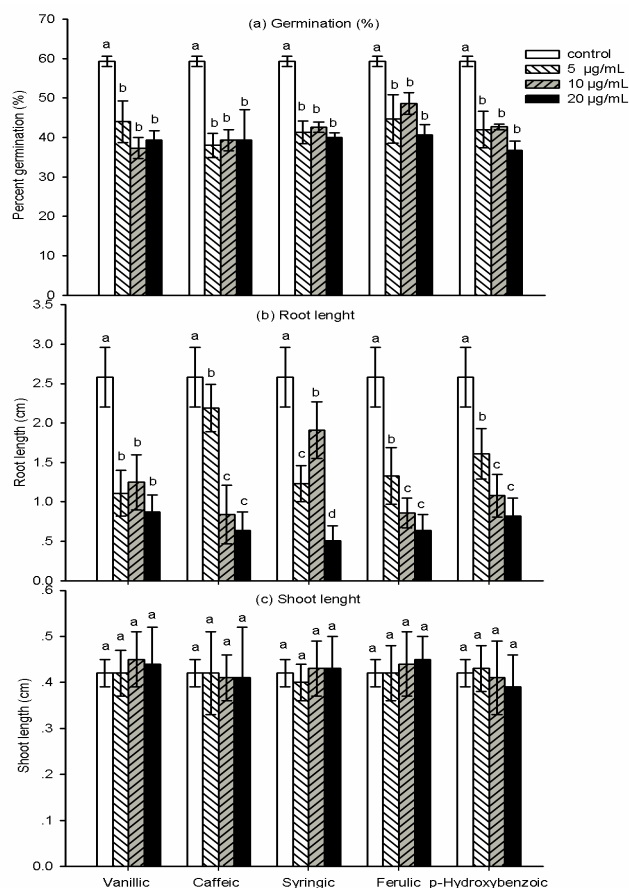


Figure 4. Effects of phenolic acids concentrations on seedling growth of *A. paniculata*. Different letters above bars indicate significant differences among treatments ($P < 0.05$) according to Duncan's multiple range tests.

Table 1. Phytotoxic effects of Andrographolide extracts against the seed germination and seedling growth of *A. paniculata*.

Concentration (mg/mL)	Germination (%)	Root length (cm)	Shoot length (cm)
0	58.0±5.77 a	4.13±1.13 a	1.07±0.34 a
0.01	53.3±4.05 a	3.72±0.86 a	0.97±0.21 a
0.05	47.3±1.33 a	2.53±0.97 b	0.93±0.16 a
0.1	54.0±3.05 a	2.81±0.88 b	1.01±0.15 a
0.2	49.3±1.76 a	2.99±0.51 b	0.95±0.15 a
0.5	57.3±1.76 a	2.21±0.59 b	0.87±0.18 b
1.0	61.3±3.33 a	2.02±0.57 b	0.85±0.32 b
2.0	66.7±4.05 a	0.60±0.07 c	0.85±0.14 b

Different letters in the same column indicate significant differences among treatments ($P < 0.05$) according to Duncan's multiple range tests.

CONCLUSIONS

The aqueous extracts of leaves, stems and replanted soil of *A. paniculata* inhibited the seed germination and seedlings root growth. Higher concentrations of andrographolide, ferulic acid, vanillic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid were found in the *A. paniculata* replant soils than in uncultivated soils. In bioassay, the andrographolide and the five phenolic compounds showed toxicity to seed germination and seedling growth of *A. paniculata*. Addition of activated carbon to the growth substrate in pots, significantly improved the growth of *A. paniculata* by enhancing biomass and chlorophyll content. These results suggested that the autotoxic potential of the root exudates released by *A. paniculata* may contribute to its replanting problem.

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