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Autotoxicity potential of cotton tissues and root exudates and identification of its autotoxins

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ABSTRACT

The autotoxicity potential of cotton shoot extract, root extract and root exudate was evaluated on germination and growth of its own seedlings. The potential phytotoxic substances responsible for this activity were identified. The shoot and root tissue extracts at 20 to 80 mg/mL inhibited all growth parameters, enzyme activities (SOD, POD, MDA) and root activity of cotton seedling. However, the growth of cotton seedling was enhanced at the lowest concentration of 10 mg/mL. Four phenolic acids (*p*-hydroxybenzoic, ferulic, gallic and vanillic acid) were identified in aqueous extract of plant parts and root exudate by HPLC. The amounts of the 4- phenolic compounds in different cotton plant parts followed the order: shoot extract > root extract > root exudate.

Key words: Antioxidant enzyme, autotoxicity, cotton, extracts, germination, identification, phenolic compounds, root, root exudates, seedling growth, shoot.

INTRODUCTION

A variety of secondary plant metabolites are released into the soil, either as exudates from living plant tissues or by decomposition of plant residues (19). Some of these substances may be associated with allelopathy, and play an important role to interfere with physiological processes by producing the stimulatory and inhibitory effects on native plants in natural plant communities (10,13). Phenolic acids are important allelopathic compounds that inhibit seed germination, plant growth and other physiological processes which result in changes in floristic composition within a plant community and dominance of one plant species over others (2,3,4,7,8,31). Plants contain thousands of natural products, but not all are implicated as being allelopathic (19). Autotoxicity is a complicated problem in agricultural production. To identify and quantify compounds contained in plant extracts or residues is an important part of the process of discovering agents of allelopathy.

Root extracts and root exudates of tea, tomato, taro, soybean, cucumber and watermelon are known to inhibit their own growth (6,21,22,27,28,32). Our previous studies showed that continuous monocropping of cotton drastically inhibited the growth and physiological processes of cotton, where the effect was enhanced with the cotton continuous cropping years in the field conditions (12). However, little information is

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available about autotoxicity of cotton fresh aqueous extract of different plant tissues including leaves, stems and roots and their main phenolic compounds.

This research aimed to identify and quantify the phenolic compounds by HPLC and determine autotoxicity effects of aqueous extracts and root exudate from cotton plants through Petri dish and pot tests. This research will promote a better understanding of allelopathy mechanisms in the natural and agricultural ecosystems through investigating the allelopathic effect and quantification of causative allelochemicals.

MATERIALS AND METHODS

Identification and quantification of phenolic compounds

Phenolic acids were identified in shoot aqueous extract, root aqueous extract and root exudate of cotton using HPLC by modified method of Batish *et al.* (5). The standard phenol compounds used for HPLC analysis were purchased (Sigma Co., USA) as high purity standards and the solvents used were chromatogram grade. Phenolic compounds were identified using an HPLC system (Waters, USA). The column was ODS-C18 (150 mm×4.6 mm, 5 µm, Agilent Technologies, USA). The mobile phase A consisted of 2% acetic acid, 9% acetonitrile in water and the mobile phase B consisted of 80% acetonitrile in water (mobile phase A: mobile phase B=9:1)with a flow rate of 1 mL min^{-1}. The wavelength of UV detector was set at 275 nm. Phenolic compounds from each fraction were identified by retention times or standard addition, and their amounts were calculated by comparing peak areas with those of standards.

Test plant materials and bioassays

Two independent experiments were carried out.

Experiment I: Forty g of air-dried and comminuted plants (shoot and root respectively) of cotton were extracted by soaking in 1L distilled water at room temperature for 36 h in a shaker. The aqueous extract was filtered through two layers of filter paper. Each stock extract was diluted appropriately with sterile distilled water to get the final concentrations of 10, 20, 40 and 80 mg/mL. The pH of extracts was adjusted to 6.5 with 1 N HAc, while mannitol was added to constant osmotic potentials of 23.4 kPa (24).

Cotton (*Gossypium hirsutum*) was chosen as the test plant. All seeds were sterilized with 3% (v/v) hydrogen peroxide solution for 5 min. Twenty seedlings of each test species were evenly spaced on two layers of filter paper in each pre-sterilized open 9-cm Petri dish. Five milliliters of aqueous extracts (concentrations of 10, 20, 40 and 80 mg/mL) were added to each Petri dish. Distilled water was the control. There were three replicates. Then the Petri dishes were placed in a plant growth chambers, maintained at a 14/10 h light/dark regime and at 26±2°C. After seven days, germination rate, root length, shoot length and fresh weight of seedlings were measured.

Experiment II: Roots of cotton seedlings were collected, weighed and immediately frozen in liquid nitrogen and stored at −84°C until biochemical analysis.

Biochemical Assay

Enzyme extraction and assay: 0.5 g root tissues were placed in a mortar and crushed to a slurry with 5.0 mL phosphate buffer (pH 7.8), followed by centrifugation at 10 000 × g for 10 min at 4°C, and the supernatant was used for enzyme assays. Superoxide dismutase (SOD) activity was measured as per Madamanchi (14). Crude extract was added to a reaction solution (3 mL) containing 50 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4) at pH 7.8, 0.1 mM EDTA, 13 mM methionine, 2 µM riboflavin and 75 µM 1-(4,5-dimethylthiazol-2-yl)- 3,5- diphenyl-formazan (MTT). The reaction was started by exposing the mixture to cool white fluorescent light (Hitachi Co., Japan) at photosynthetic photon flux of 50 µmol m^{-2} s^{-1} for 15 minutes. Then the light was switched off, the tubes were stirred and the blue colour was measured at 560 nm. Guaiacol peroxidase (POD) activity was assayed in a reaction solution (3 mL) containing 50 mM phosphate buffer at pH 7.0, 1% guaiacol, 0.4% H_2O_2 and 10 µL crude extract. Increase in the absorbance due to oxidation of guaiacol was measured at 420 nm.

Determination of malonyldialdehyde (MDA) content: Lipid peroxidation was determined in terms of 2-thiobarbituric acid (TBA) reactive metabolite, chiefly malonyldialdehyde (MDA). Cotton leaves were extracted with 50 mmol/LPBS (pH 7.0). After centrifugation at 2000 × g, 0.3 mL supernatant was added to 3 mL 20% TCA (including 1% TBA). The solution was quickly cooled after water-heating at 90°C. Following centrifugation at 10000 × g for 10 min, the absorbance of the supernatant was measured at 532 nm. The level of lipid peroxidation was expressed as µmol of MDA formed using an extinction coefficient of 155 mmol/cm.

Assay of root vigour: The vigour of roots was estimated by method of Arnon (1). 0.5 g root tissues were placed in a beaker, and watered with equivalent mixture 10 mL (TTC solution and phosphoric acid buffer), roots were fully immersed in equivalent mixture preserved for 3 h at 37°C, then watered with vitriol 2 mL (1 mol/L) to stop the reaction. At the same time a blank experiment was made (after addition of root samples, water with sulphuric acid, other operating as same as above). The roots were taken out, ground with ethyl acetate (3-4 mL) to get red extract, then it was added to tube and ethyl acetate was added to make the total volume of 10 mL, followed by measurement at 485 nm, with blank test, check the standard curve, you could calculate the amount of tetrazolium reduction.

Statistical analysis

The data were analyzed using Microsoft Office Excel 2003 and SAS 6.12 programs. Means, least significant differences (LSD) of 5% level were calculated using SAS software (Vol.6.12).

RESULTS AND DISCUSSION

Autotoxicity of aqueous extracts to seedling growth

The aqueous extracts from cotton shoot and root were autotoxic to the germination rate, radicle elongation, shoot height, number of root and dry weight of cotton seedling (Table 1). Significant inhibitory effects were observed at all test concentrations and the inhibition increased with the increase in concentration of aqueous extracts. The extracts from 20 to 80 mg/mL of shoot and root tissues inhibited all the growth parameters of cotton seedlings, however, the lowest concentration (10 mg/mL of root tissue) stimulated the seedlings' growth. In all concentrations applied, germination and seedling growth were more affected by cotton shoot extract than by root extract. At highest shoot extract concentration (80 mg/mL), germination rate, radicle elongation, shoot height, number of root and dry weight were decreased by 18.2, 34.4, 39.2, 43.6 and 20.3%. But the root extracts at 80 mg/mL caused 9.1, 17.9, 24.3, 27.2 and 16.7% reductions, respectively. The inhibitory potential of shoot and root aqueous extracts from cotton plant on the germination rate, radicle elongation, shoot height, number of root and dry weight of cotton seedling was taller than average at high concentrations(40 mg/mL and 80 mg/mL), but, at low concentration (10 mg/mL and 20 mg/mL) was lower than average values.

Table 1. Inhibitory effects of aqueous extracts from cotton plant on its seedling growth

Plant Part	Aqueous Extract Conc (mg/mL)	Inhibition (%) over control				
		Germination	Radicle length	Shoot height	Root numbers	Dry weight
Shoot	80	18.2±2.33	34.4±3.6	39.2±4.12	43.6±3.73	20.3±1.68
	40	12.3±1.43	19.6±2.8	24.5±2.61	24.7±2.19	9.8±0.15
	20	4.4±0.28	5.7±0.73	8.1±1.12	-8.1±0.87	4.5±0.38
	10	-3.4±0.16	-8.4±0.52	-6.9±0.38	-19.4±2.66	-3.8±0.29
	Mean	7.9±1.05	12.8±1.91	16.2±2.05	10.2±2.36	7.7±0.63
Root	80	9.1±1.21	34.4±3.6	39.2±4.12	43.6±3.73	20.3±1.68
	40	5.4±0.37	19.6±2.8	24.5±2.61	24.7±2.19	9.8±0.15
	20	-3.7±0.16	5.7±0.73	8.1±1.12	-8.1±0.87	4.5±0.38
	10	-6.4±0.46	-8.4±0.52	-6.9±0.38	-19.4±2.66	-3.8±0.29
	Mean	1.1±0.55	12.8±1.91	16.2±2.05	10.2±2.36	7.7±0.63

Many autotoxins had been identified from the root exudates, plant tissues and plant leachates (20,28), which altered the plant growth by influencing various physiological processes (17,32). Secondary compounds with allelopathic potential were produced in different plant tissues including leaves, stems, roots and seeds (16). However, the secondary metabolites varied in chemical-composition, concentration and varied with different plant parts and plant species (9). Reinhardt and Bezuidenhout (18) demonstrated that leaves were the most consistent source of phytotoxic chemicals, while fewer and less potent toxins were present in roots, which was consistent with our study. The results of this study indicated that the shoot aqueous extracts were more inhibitory to seed germination and seedling growth than root aqueous extracts.

Inhibitory effects of plant exudates to POD activity

POD activity of cotton seedling was greatly inhibited by all concentrations of shoot and root exudates. The inhibition increased with the increment in the concentration of shoot and root exudates (Figure 1). The greatest inhibition was obtained at the highest concentration. Aqueous shoot and root exudates at 10 and 20 mg/mL were inhibitory to

POD, but increased the POD activity at 40 and 80 mg/mL. POD activities were only 37.7 and 52.3% of the control at 40 and 80 mg/mL for the plants of cotton treated by shoot extracts, 31.9 and 33.2% of the control for the root extract treatment, respectively. In general, shoot extracts produced greater inhibition in POD than root extracts.

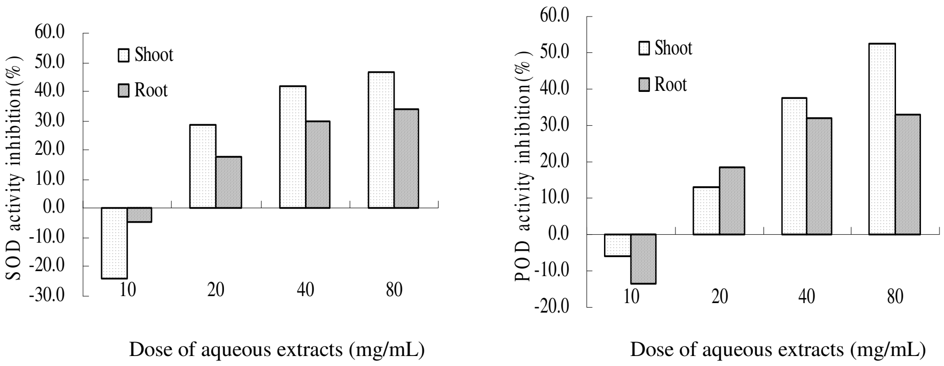

Figure 1. Inhibitory effects of cotton aqueous extracts on SOD and POD activity of its seedlings

Inhibitory effects of plant exudates to SOD activity

All applied doses of cotton shoot and root extracts diminished the SOD activity, except the lowest dose (Figure 1). As the highest extract concentration was compared to the control the shoot extract, the reduction were approximately 46.7% and the root extract 33.8% (Figure 1). In addition, the inhibitory effects of shoot extract were higher than that of root extract. Twenty and 40 mg/mL concentrations caused 28.7 and 42.2% inhibition in shoot extract, while there was a nearly 29.7 and 33.8% reduction caused by root extract.

Inhibitory effects of plant exudates to root activity

Root activity of cotton seedling was significantly inhibited by water-extracted solution of cotton plant parts (Figure 2). The extents of the inhibitory effects varied with the concentrations of the water-extracted solutions. The extracts from 20 to 80 mg/mL in shoot and root tissue inhibited the root activity of tested plants, however, the lowest amount (10 mg/mL of cotton tissue), significantly accelerated the elongation of roots. The inhibitory effects by shoot extracts on root activity were 32.7, 41.1 and 46.7% at 20, 40 and 80 mg/mL, while those by root extracts were 10, 21.6 and 38.2%, respectively.

Inhibitory effects of plant exudates to MDA content

The aqueous extracts from cotton tissues were inhibitory to the contents of MDA of tested plants (Figure 2). Significant inhibitory effects were observed at all concentrations and increased with the increase in concentrations of aqueous extracts. MDA contents of test plants were more sensitive to shoot extracts than root extracts at all concentrations . The root extract at 10 mg/mL did not inhibit the MDA contents of cotton and the MDA contents increased from 11.9, 12.8 to 21.8% as the extract concentrations

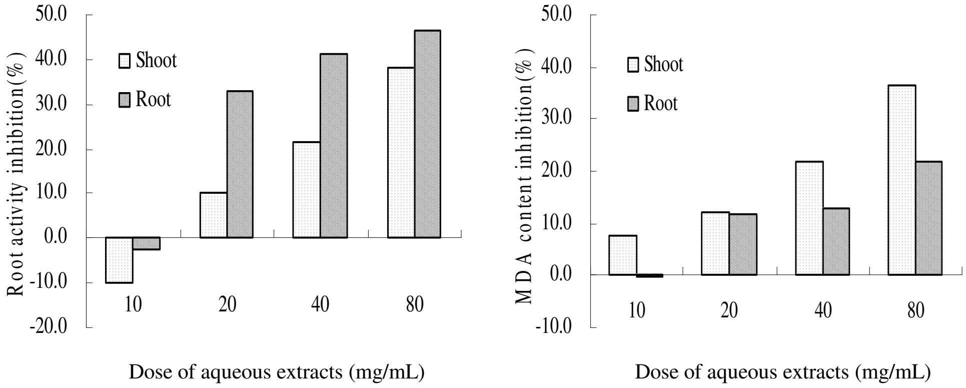

Figure 2. Inhibitory effects of cotton aqueous extracts on root activity and MDA content of its seedlings

increasing from 20 to 80 mg/ml. The cotton shoot extracts at 10, 20, 40 and 80 mg/mL caused 7.8, 12.0, 21.9 and 31.5% reductions in MDA contents of test plants.

The seed germination and seedling growth involved complex transformations and their integration at biochemical level. Different metabolic processes became active during germination, which resulted in degradation of reserve materials translocation from the storage tissue and their translocation to the growing embryo to produce new compounds for seedling establishment (23). This meant that the effects observed on these physiological parameters by plant extracts would be due to organic chemical compounds in solution. The allelopathic phenomena could be, at least partially, responsible for the observed effects. It was well known that environmental stresses including allelochemicals could inhibit plant growth partly by increased generation of reactive oxygen species (ROS) (26,29). Meanwhile, plants also contain ROS-scavenging enzymes such as SOD and POD, to eliminate the harmful effects of ROS. All our results suggested that root viability, SOD and POD activities in seedlings could be inhibited by autotoxic substances. It was in agreement with our previous study on cotton, in which root TTC reduced activity, POD activity and MDA content were inhibited by monocropping, but it could be alleviated by activated charcoal (12)

Identification and quantification of the allelochemicals

To identify the autotoxins of cotton plants, the contents of phenolic acids in shoot aqueous extract, root aqueous extract and root exudate were tested by HPLC (Table 2). Four phenolics (*p*-hydroxybenzoic, ferulic, gallic and vanillic acid) were identified in aqueous extracts of plant parts and root exudate. The amount of *p*-hydroxybenzoic acid (26.21-42.39 μg/g) was maximum in shoot and root, followed by gallic acid (4.31-40.65 μg/g) and minimal in ferulic acid (3.76-12.48 μg/g). In three samples of leachate, the total content of four phenolic acids in shoot aqueous extract was higher than root aqueous extract and root exudate (Figure 3). The contents of phenolic substances in three samples of leach liquor were 103.52, 70.48 and 47.76 ug/g, respectively.

Table 2. Quantitative determination by HPLC analysis of some phenolic compounds in plant parts and root exudates of cotton

Phenolic compounds	Phenolic compounds concentrations (μg/g)			
	Aqueous extracts		Root exudate	Total
	Shoot	Root		
p-hydroxybenzoic acid	42.39±5.26	32.17±4.33	26.21±1.75	100.77
Ferulic acid	5.47±0.66	3.76±0.38	12.48±1.16	21.71
Gallic acid	40.65±6.06	25.94±2.46	4.31±0.36	70.9
Vanillic acid	15.02±1.82	8.62±1.21	4.77±0.45	28.41
Total	103.53	70.49	47.77	

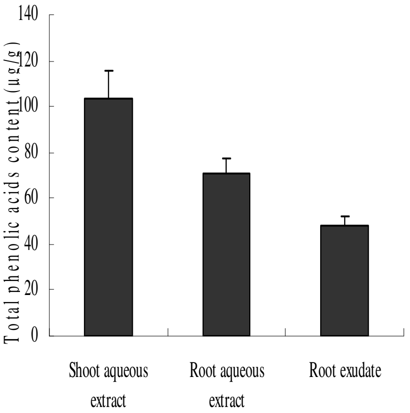

Figure 3. Total phenolic acids contents in the cotton shoot aqueous extract, root aqueous extract and root exudate

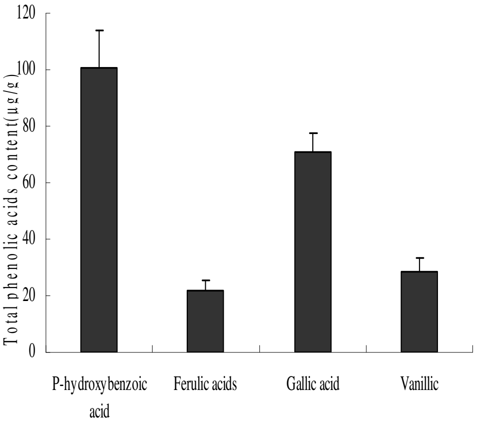

Figure 4. The contents of four phenolic compounds from cotton tissues

The amount of phenolic compounds from the root secretion was different than aqueous extract of cotton tissues (Figure 4). In aqueous extract of plant, the content of gallic acid was highest, but, in root exudates, the content of gallic acid was lowest. It was 10.61% and 16.62% in shoot and root aqueous extract, respectively. While the content of ferulic acid was highest in the root exudate, which was 2.3 and 3.3 times higher than in aqueous extract of shoot and root of the plant. The research on relative content of various phenolic compounds in different organs and position of cotton plant showed that the root secretion was the main source of ferulic acid, while gallic and vanillic acids mainly came from the aqueous extract of plant, whereas the content of the *p*-hydroxybenzoic acid was higher in both. The contents of four phenolic compounds in the shoot and root of the plant and the root secretion followed the order :*p*-hydroxybenzoic > gallic > vanillic > ferulic acid .

Allelochemicals are species-specific and concentration-dependent. Allelopathic activities of phenolic compounds may be dependent upon the synergistic or additive effects of the types of constituents present at different concentrations (15,25). Several phenolic compounds at different concentrations inhibited the cotton seed germination, seedling growth, protective enzyme system and the malondialdehyde (MDA) content of different species. These results were in agreement with some previous report (11,12,26,29,30). In these studies, 4 autotoxins (*p*-hydroxybenzoic, ferulic, gallic and vanillic acid) were identified in the plant aqueous extracts and root exudates. These results suggested that the cotton cannot be cropped continuously due to accumulation of phenolic acids in the soil, that reduces the productivity of cotton with years.

CONCLUSIONS

The cotton plants exert autotoxic potential by exuding autotoxic substances, besides their allelopathic potential to affect the plant growth of other species. The cotton seedling growth parameters and physiological parameters were inhibited by the phytotoxic substances, which include *p*-hydroxybenzoic acid, gallic, ferulic and vanillic acid accumulated in the tissues. A large portion of these easily soluble and degradable compounds entered the soil with rain, dew and fog, and accumulated in the surface soil layer (6). But, allelopathic effects could not readily be discriminated from the environmental factors under field conditions. More research about the fate and behaviour of phytotoxic substances, their fluctuation in the soil as well as the mechanism of action are needed.

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