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Phenolic acids contents and allelopathic potential of 10-cultivars of alfalfa and their bioactivity

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ABSTRACT

By high performance liquid chromatography (HPLC) we determined the content of 6-phenolic acids (*p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, ferulic acid and cinnamic acid) in fresh aerial parts and roots of 10-cultivars of alfalfa. The results showed that the concentrations and compositions of phenolic acids depended on the aerial part or roots of alfalfa. Bioassays were conducted with leaf litter leachates from all these cultivars on the seeding growth of *Festuca arundinace* and *Sorghum sudanense*. The growth of *F. arundinace* and *S. sudanense* was inhibited variably by different cultivars of alfalfa. At 50 mg/mL, the inhibitory effects of leaf litter leachates were higher on roots than on shoots. Our results demonstrate that the content of phenolic compounds and the allelopathic potential of 10-cultivars of alfalfa are differed from each other.

Key words: Alfalfa, allelochemicals, allelopathic compounds, bioassay, biomass, cultivars, *Festuca arundinace*, HPLC, leachate, leaf litter, phenolic acids, root, seedling growth, shoot, *Sorghum sudanense*

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is called the "Queen of the Forages" owing to the nutritional quality (5,16,17). Alfalfa is one of the major forage legume used for soil improvement and livestock feed (22,29). Its plants contains water-soluble allelopathic compounds that are autotoxic to the same species and other plants, hence, it is both allelopathic and autotoxic (8,12,24,29). Allelopathy is an important mechanism of plant competition by releasing the allelopatic compounds into environment to inhibit other plants growth and development (1,15,20,24). Autotoxicity reduces the seed germination, establishment and dry matter yield, when alfalfa is replanted before the autotoxic compounds are decomposed (3,14). To overcome the problem of autotoxicity, the recommended way is to delay alfalfa sowing after next alfalfa crop for at least 2-weeks or

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under certain condition up to 2-years (3,9).

Phenolic acids are of great significance in allelopathy or autotoxicity (18,22,23). They are the main groups of phytotoxic substances associated with alfalfa allelopathy (2,4). Among nine phenolic compounds (caffeic acid, *trans*-cinnamic acid, hydrocinnamic acid, coumarin, ferulic acid, m-coumaric acid, o-coumaric acid and *p*-coumaric acid) assayed for their phytotoxicity on root growth of alfalfa, coumarin, trans-cinnamic acid and o-coumaric acid at 10^{-3} M are most inhibitory to the root growth of alfalfa (4). Root exudates of alfalfa seedlings were extracted by dichloromethane from the rhizosphere soil and identified by Gas Chromatography-Mass Spectrometer (GC-MS) (28). The results showed that the main compounds in root exudates of alfalfa were: phenolic acids, straight-chain alkanes, alcohols, cycloalkanes, methyl thiophenol, benzene, phenols, esters, alkenes, heterocyclic aniline, naphthylamine and polycyclic aromatic compounds (28). The allelopathic effects of alfalfa, competitive ability and harvesting regime may play an important role in suppressing the seedling growth of *A. vulgaris* (16). In greenhouse and laboratory experiments the alfalfa shoot biomass was more inhibitory than under-ground alfalfa biomass (16). Above-ground alfalfa biomass was very inhibitory to the seed germination and seedling growth of *Artemisia vulgaris*, may be due to some allelopthic compounds (16).

Limited information exists, about the allelopathic phenolic compounds from the root exudates and comparison of the allelopathic potential of different cultivars of alfalfa. This study aimed (a) to compare, identify, quantity and the content of allelopathic phenolic acids in the extracts from aerial part and roots of 10-cultivars of alfalfa and (b) to determine the phytotoxicity of leaf litter leachate of 10-cultivars of alfalfa on the seedling growth of *Festuca arundinacea* Schreb. and *Sorghum sudanense* (Piper) Stapf.

MATERIALS AND METHODS

Seeds of ten alfalfa varieties (Hunterriver, Longdong, Aohan, Surprise, Pioneer, Victoria, Post mark, WL-525HQ, WL903 and WL-343HQ) were purchased from the Beijing Rytway Eco-technology Co., Ltd. While the seeds of *Festuca arundinacea* Schreb. and *Sorghum sudanense* (Piper) Stapf. were purchased from Guangzhou Seed Company Guangzhou, China. The *p*-Hydroxybenzoic acid (≥ 99.0%), caffeic acid (≥ 98.0%), *p*-coumaric acid, chlorogenic acid (≥ 98.0%), ferulic acid(≥ 97%) and cinnamic acid (≥ 99%) were purchase from Sigma-Aldrich.

Chemical analysis of leachates

To evaluate the detection limits, all the phenolic acids were dissolved separately in methanol (≥ 99.9 %) at 1000 µg/mL concentration as stock solutions. A sequence of 20, 40, 60, 80 and 100 µg/mL concentration of the standards was obtained by diluting these stock solutions with methanol. The separation of phenolic acids was performed with an Agilent 1100 series high performance liquid chromatography (HPLC) system equipped with photodiode array detector. Instrument control and data analysis was carried out using Agilent HPLC Chemstation 10.1. The flow rate of the mobile phase was kept at 1 mL/min. Mobile phase A was water containing 25% methanol, and phase B was 75% acetic acid (pH 2.6). The temperature of column was controlled at 20°C. Injection volume was 25 µL. The detection wavelengths of diode array detection (DAD) were set at 280 nm.

For the analysis of these phenolic acids, fresh aerial part or roots (100 g) of ten cultivars of alfalfa collected from our Experimental Farm were soaked in 200 mL distilled water for 48 h at room temperature (25 ± 2°C) to get 0.5 g/mL aqueous leachates, respectively. The leachates were filtered through 0.45 µm nitrocellulose membrane filters and stored at 4°C until further use. 25 µL of each aqueous leachate were analyzed using Agilent HPLC Chemstation 10.1. by the same method decribes above. Content of these phenolic acids in the aqueous leachates were calculated by comparing the peaks areas of samples with those of standard compounds (26).

Bioassays

Fresh leaves of ten cultivars of alfalfa were collected and air-dried at room temperature (July 2016). The allelopathic effects of leaf litter leachate from ten cultivars of alfalfa were evaluated by using the sandwich method (7). Ten, 30 or 50 mg of dried leaf material was placed between two layers of semi-solid agar (0.5% w/v) in 50 mL beakers (7). The *Festuca arundinace* and *Sorghum sudanense* are commonly used test species in allelopathic studies. Five germinated seeds of *F. arundinace* and *S. sudanense* were vertically placed on the surface of the water agar and then the beaker was covered with plastic film and placed in the growth chamber (25 ± 2 °C, dark condition) (7,21). Shoot heights and root lengths of seedlings were recorded 4 days after incubation. All treatments were replicated thrice.

Statistical analysis: Allelopathic potential of ten cultivars of alfalfa on the target plants and content of phenolic acids of ten cultivars of alfalfa were analyzed using one-way ANOVA followed by the Duncan's multiple range tests. Effects of different cultivars and concentration of alfalfa on plant growth were analyzed using two-way ANOVA tests by SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

I. Chemical constituents of alfalfa aerial parts

The quantities of exuded allelochemicals phenolic acids from aerial parts of alfalfa ranged : *p*-hydroxybenzoic acid (13.62 to 39.18 µg/g), caffeic acid (11.78 to 31.55 µg/g), chlorogenic acid (48.81 to 74.80 µg/g), *p*-coumaric acid (1.56 to 5.96 µg/g), ferulic acid (3.90 to 14.30 µg/g), cinnamic acid (1.32 to 21.86 µg/g), and total phenolic acids (115.44 to 153.96 µg/g) of fresh weight (Table 1). *p*-Hydroxybenzoic acid contents of the extracts from the cultivar Surprise, Victoria and WL-903 were 39.18, 36.85 and 35.97 µg/g, respectively, higher than those from other alfalfa cultivars (Table 1).

Table 1. The phenolic acids content of aqueous extract from aerial parts of 10-cultivars of alfalfa (µg/g FW)

Cultivars	*p*-Hydroxybenzoic acid	Caffeic acid	Chlorogenic acid	*p*-Coumaric acid	Ferulic acid	Cinnamic acid	Total Phen -olic acids
Hunterriver	26.09 cd	23.03 b	52.08 c	1.56 d	8.78 bc	3.90 e	115.44 c
Longdong	35.29 b	28.80 a	61.65 b	3.39 bc	11.22 b	13.60 b	153.96 a
Aohan	28.35 c	21.53 bc	52.30 c	5.96 a	7.42 cd	1.32 e	116.86 c
Surprise	39.18 a	11.78 d	62.60 b	3.65 bc	14.30 a	8.00 d	139.52 b
Pioneer	24.40 d	31.55 a	51.46 c	2.82 cd	5.35 de	21.86 a	137.44 b
Victoria	36.85 ab	22.52 bc	60.89 b	4.63 ab	5.81 de	10.49 c	141.19 b
Post mark	25.08 cd	21.05 bc	74.80 a	3.39 bc	8.93 bc	3.14 e	136.37 b
WL-525HQ	28.26 c	27.72 a	48.81 c	2.78 cd	7.35 cd	2.48 e	117.41 c
WL-903	35.97 ab	18.52 c	74.73 a	2.73 cd	3.90 e	1.88 e	137.74 b
WL-343HQ	13.62 e	30.70 a	61.45 b	4.22 bc	13.98 a	7.55 d	131.51 b

P < 0.05 Using Duncan's multiple range test) among all treatments are indicated by different letters

II. Chemical constituents of alfalfa aerial roots

The highest contents of the *p*-Hydroxybenzoic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, cinnamic acid and total phenolic acids were in the aqueous extract from roots of alfalfa varieties viz., WL-525HQ (13.05 µg/g), WL-525HQ (7.23 µg/g), WL-343HQ (22.97 µg/g), WL-343HQ (8.35 µg/g), Surprise (3.92 µg/g), Longdong (3.10 µg/g) and Longdong (50.46 µg/g), respectively (Table 2). Many studies have reported the differences in allelochemicals among extracts from various tissues of alfalfa (4,19,22,26). The quantities of exuded allelochemicals varied with the specific compound and ranged: *p*-hydroxybenzoic (2.3 to 18.6 µg/L), vanillic (0.6 to 17.5 µg/L), cis-*p*-coumaric (0.1 to 4.9 µg/L), syringic (0.0 to 52.7 µg/L), cis-ferulic (0.33 to 12.7 µg/L), trans-*p*-coumaric (1.5 to 20.5 µg/L), and trans-ferulic acids (1.6 to 23.4 µg/L) of water/agar. Acidic fractions were isolated as per Xuan *et al.* (26) and the inhibitory effects of the acidic fraction from the extracts of 3-alfalfa cultivars (Batasu, Rasen, and Yuba) were variable on the seedlings growth of alfalfa and rice (26). The extract from Batasu was least inhibitory and that of Rasen was most inhibitory (26). These esults clearly indicated that the phenolic acids in the aqueous extracts from roots were variable in alfalfa cultivars.

Table 2. The phenolic acids content of aqueous extract from roots of 10-ten cultivars of alfalfa (µg/g FW)

Cultivars	*p*-Hydroxybenzoic acid	Caffeic acid	Chlorogenic acid	*p*-Coumaric acid	Ferulic acid	Cinnamic acid	Total phen -olic acid
Hunterriver	6.29 bc	3.10 d	20.43 abc	3.36 cd	3.70 a	1.43 bcd	38.30 de
Longdong	11.35 a	6.67 ab	21.34 ab	4.80 bc	3.20 ab	3.10 a	50.46 a
Aohan	7.17 b	2.79 d	18.02 c	3.04 d	2.51 bcd	1.07 d	34.60 e
Surprise	12.94 a	3.88 cd	18.21 c	7.33 a	3.92 a	1.14 d	47.42 ab
Pioneer	12.17 a	5.30 bc	19.40 bc	3.79 cd	1.15 e	1.86 bcd	43.67 bc
Victoria	12.17 a	4.19 cd	15.00 d	5.47 b	1.81 de	2.00 bc	40.64 cd
Post mark	4.45 c	3.03 d	19.85 bc	4.14 bcd	2.08 cde	2.16 b	35.71 de
WL-525HQ	13.05 a	7.23 a	19.03 bc	3.38 cd	1.19 e	1.32 cd	42.20 bc
WL-903	8.40 b	3.91 cd	19.60 bc	5.64 b	1.91 de	1.15 d	40.62 cd
WL-343HQ	6.30 bc	4.70 c	22.97 a	8.35 a	3.03 abc	1.47 bcd	46.80 ab

P < 0.05 Using using Duncan's multiple range test) among all treatments are indicated by different letters.

III. Bioassays results

The "Sandwich" bioassays (6,21) were done to evaluate the phytotoxic effects of leaf litter leachate from ten cultivars of alfalfa on the seedling development of *F. arundinacea* (Fig. 1A and B) and *S. sudanense* (Fig. 2A and B). The leaf litter leachates from Hunterriver, Longdong, Aohan, Surprise, Pioneer, Victoria, Post mark, WL-525HQ, WL-903 and WL-343HQ at 10 mg/mL inhibited the root length of *F. arundinacea* seedlings by 11.1, 5.0, 11.6, 7.4, 11.3, 19.4, 19.7, 20.4, 16.2 and 13.9%, respectively (Fig. 1A). The same trends were observed in root lengths of *S. sudanense* (Fig. 2A). The root growth of *F. arundinacea* and *S. sudanense* was more sensitive than shoot growth. The root length of *F. arundinacea* were inhibited by 46.6, 57.8, 51.4, 54.8, 52.4, 66.9, 68.1, 55.1, 68.0 and 54.5% when leaf litter leachate from Hunterriver, Longdong, Aohan, Surprise, Pioneer, Victoria, Post mark, WL-525HQ, WL-903 and WL-343HQ were applied at 50 mg/mL, respectively (Fig. 1A). The magnitude of inhibition from the leaf litter leachate of alfalfa cultivars in root and shoot growth of *F. arundinacea* (Fig. 1) and *S. sudanense* (Fig. 2) with increased with increase in concentration i.e. concentration dependent.

The different cultivars of alfalfa significantly inhibited the root length ($F = 8.408$, $P < 0.001$) and shoot height ($F = 2.236$, $P < 0.001$) of *F. arundinace*. The root length ($F = 497.495$, $P < 0.001$) and shoot height ($F = 843.330$, $P < 0.001$) of *S. sudanense* were significantly affected by the different concentrations of leaf litter leachate from different cultivars of alfalfa ($F = 2.208$, $P = 0.003$; $F = 1.970$, $P = 0.010$, respectively). The root extracts promoted the root growth at lower concentration but inhibited at higher concentration (10). Similarly, the alfalfa extracts inhibited the growth of root and seedling growth of cotton and the inhibition was stronger with the increased extract concentrations (27). The allelopathic potential of aqueous extracts of roots on the seeding and roots of radish differed among the alfalfa varieties: Sanditi, Eureka, Alize and Alfaqeen (11). The aqueous extracts from Derby, Sanditi, Eurek, Baralfa, Alfaqueen and Sadie at 0.1 g/mL inhibited the germination and seeding growth of *Dactylis glomerata* (6). The aqueous extracts from Sanditi at 5, 7.5 and 10 % decreased the germination rate of *D. glomerata* by 5.0, 18.1 and 51.9 % than control respectively (6). Likewise, the shoot height of *D. glomerata* was significantly inhibited by 31.9, 14.4 and 28.9 % when the aqueous extracts from Derby at 5, 7.5 and 10 %, respectively (6). The aqueous extracts form alfalfa stem and leaf (0.1 g/mL) inhibited the seed germination rate, seedling length and root length of *Bromus inermis* by 35.27, 11.63 and 64.09 %, respectively (18). Of all the 23 alfalfa varieties, the allelopathic activities of WL324, Aohan, Gannong No.1 and Gannong No.4 were inhibitory to the seedling growth of lettuce, while, inhibition in the seedling growth of wheat was from Cannong No. 1, Gannong No. 3, Gannong No. 4 and Gongnong No. 1 varieties, (13). The highest inhibition was found in the leaf extracts from the variety "Vernal" (40 g/L) which were 17.5, 15.4 and 28.7 folds more toxic to root growth of alfalfa than stems, roots and seeds, respectively (4). Among the 9-phenolic compounds, coumarin, trans-cinnamic acid and o-coumaric acid were most inhibitory to the seedling growth of alfalfa (4). Alfalfa pellets significantly inhibited the germination and growth of weeds (*Monocholia vaginalis*, *Cyperus difformis*, *Digitaria ciliaris* and *Echinochloa orygicola*) in rice fields (25). The weeds growth inhibition by alfalfa pellet became stronger with the

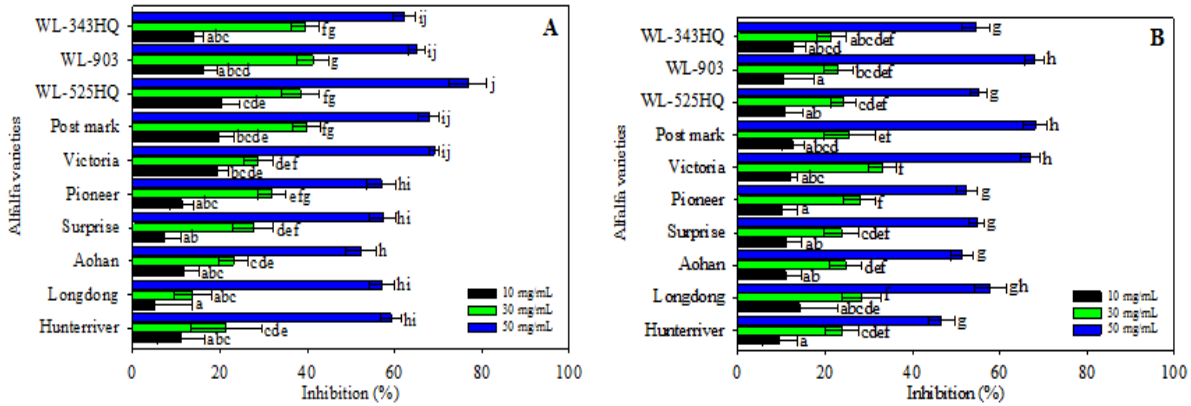

Figure 1. Phytotoxicity of leaf litter leachates of 10-cultivars of alfalfa on the root length (A) and shoot height (B) of *Festuca arundinace*. Values are means ± SE from three replicates. Significant differences ($P < 0.05$ using Duncan's multiple range test) among all treatments are indicated by different letters above bars.

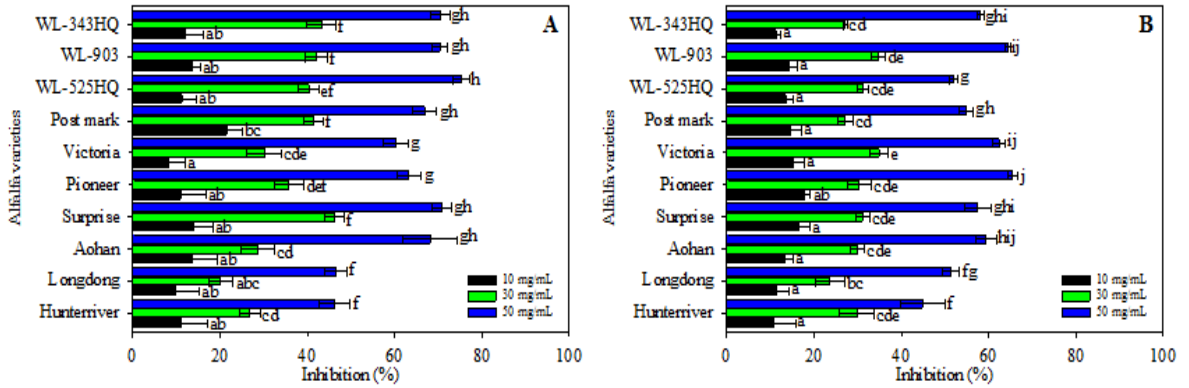

Figure 2. Phytotoxicity of leaf litter leachates of 10-cultivars of alfalfa on the root length (A) and shoot height (B) of *Sorghum sudanens*. Values are means ± SE from three replicates. Significant differences ($P < 0.05$ using Duncan's multiple range test) among all treatments are indicated by different letters above bars.

increased dose of application (25). Wu *et al.* reported that the presence of allelopathic compounds in plant tissues does not necessarily mean that these compounds can be exuded into the environment to affect the growth of its neighbouring plants (22). Our study has clearly shown the allelopathic effects of leaf litter leachates from different cultivars of alfalfa on the seedling growth of *F. Arundinacea* and *S. Sudanense.* Our results also indicated that the allelopathic inhibition rate increased with increase in leaf litter leachates concentration.

CONCLUSIONS

We identified and measured 6-phenolic acids (*p*-Hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, ferulic acid and cinnamic acid) from ten alfalfa cultivars (Hunterriver, Longdong, Aohan, Surprise, Pioneer, Victoria, Post mark, WL-525HQ, WL903 and WL-343HQ) by HPLC. The bioassay showed the allelopathic potential of different cultivars of alfalfa on the seedling growth of the test receptor plants (*F. arundinacea* and *S. sudanense*) was significantly variable. Also conducting of this experiment using soil could lead to reliable results for evaluation of allopathic effects of 10-cultivars of alfalfa on the target plants. However, more long-term research is needed to evaluate the allelopathy and autotoxicity of different cultivars of alfalfa in field conditions.

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