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Allelopathic Potency and an Active Substance from Hairy Vetch

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ABSTRACT

Background: Plant allelopathy refers to the release of chemicals from plants or microorganisms into the environment, may have direct or indirect, beneficial or harmful effects on other plants or microorganisms. When plants grow in an unfavorable environment, more allelochemicals will be secreted and the expression of allelopathic effects will increase, giving plants a certain competitive advantage. Hairy vetch is one of the most promising allelopathic crops and the aqueous extract of hairy vetch has an inhibitory effect on the root length and seedling height of grass crops. The current study aimed to study the allelopathic effect of hairy vetch on alfalfa, and exploring an ecological method to remove the root system of alfalfa.

Methods: In this experiment, the allelopathic effects of the seeds, stems and leaves, roots extracts and root exudates (0, 3, 6, 9 and 12 mg·mL^{-1}) on the seed germination and seedling growth of alfalfa were evaluated. And the main allelopathic substances from the stem and leaf extract were isolated and identified using high performance liquid chromatography- mass spectrometry (HPLC-MS).

Result: The results showed that all of the extracts can inhibit alfalfa seed germination and seedling growth and stem and leaf extract had the strongest inhibitory effect, especially for inhibiting the root growth. A main allelochemical substance, o-coumaric acid, was screened out and the root length of alfalfa was completely inhibited at 1.6 mg·mL^{-1} of o-coumaric acid. The findings of these experiments show hairy vetch has strong allelopathic effect on alfalfa and o-coumaric acid is a chemical growth inhibitor.

Key words: Alfalfa, Allopathic potency, Hairy vetch, Inhibitory activity, O-coumaric acid.

INTRODUCTION

Plant allelopathy refers to the release of chemicals from plants or microorganisms into the environment that may have direct or indirect, beneficial or harmful effects on other plants or microorganisms (Rao, Singh *et al*. 2018). The modes of action of allelochemicals mainly include leaching, root secretion, volatilization and plant decomposition (Antje *et al.,* 2018). Allelochemicals are found in various plant organs, including plant roots, stems, leaves, flowers and seeds (Shiraishi *et al*., 2002). Plant allelochemicals can alter the affected plants by physiological, biochemical, or molecular aspects of targeted growth, including seed germination, stomatal activity, water and nutrient uptake, membrane function, photosynthesis, respiration and enzymatic activity (Espinosa-Rodríguez *et al*., 2017).

A previous study evaluated hairy vetch is the most promising allelopathic crop compared with 53 species of plants (Fujii 2003). Hairy vetch contains allelochemicals that selectively inhibit weeds and can thus directly inhibit field weeds to reduce the use of chemical herbicides.

These previous studies have shown that the hairy vetch plant has an allelopathic effect on weeds. Additionally, when conducted a field experiment, we found that alfalfa growth and weed growth were inhibited in the field where the hairy vetch was planted. On September 24th, 2017, the hairy vetch was interplanted in four years of alfalfa. On June 13th, 2018, the hairy vetch and the alfalfa were harvested at the same time. The reproducibility of the four years alfalfa became worse and the growth of weeds was inhibited in the field with the hairy vetch. Therefore, we hypothesized that the hairy vetch plant could have an allelopathic effect on alfalfa. In order to test our hypothesis, we carried out the following tests. The growth of alfalfa was test by hairy vetch extracts and the main allelochemical of hairy vetch was tested and further verified.

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MATERIALS AND METHODS

Plant Material

The variety of alfalfa is Suntory Alfalfa, the alfalfa seed was purchased from the Beijing Best Company. The hairy vetch was harvested in the Jiaozhou Science and Technology Demonstration Station of Qingdao Agricultural University. The hairy vetch was washed several times to remove soil and the separated into stems and leaves and roots. Seeds, stems and leaves and roots were dried in an oven at 40°C and ground into powder. Dry powder then vacuum sealed in a plastic bag and kept at 4°C.

Extraction

Seeds powder, stem and leaf powder and roots powder (100 g), were extracted separately using 1 L of 70% (v/v) aqueous methanol for 24 h (Ma *et al*., 2017). The extract of each plant powder was then filtered through one layer of

filter paper, using a vacuum pump. The residue was extracted again using 1 L of 70% methanol for 24 h and then filtrated. The two filtrates of each part were combined. The filtrates of each part of the hairy vetch and root exudates (1 L) were evaporated to dryness using a rotary evaporator at 40°C. Each crude extract was placed in the refrigerator 4°C and subsequently used for the next experiments.

Bioassay of alfalfa seed germination

Choose 1000 alfalfa seeds of uniform size and full grain. The alfalfa seeds were wrapped with gauze, the surface soil was washed under running water and soaked for 30 minutes with 0.1% sodium hypochlorite. The surface of the sodium hypochlorite was rinsed with distilled water. After disinfecting with 75% alcohol for 10 minutes, rinse the surface with distilled water. The seeds were sown in a wet sand tray in an incubator set at a temperature of 30±2°C (Cruz-Ortega *et al*., 1998).

The bioassay was conducted with five concentrations of extract (0, 3, 6, 9 and 12 mg· mL^{-1}). 5 mL of each of the different concentrations of extract were added to a petri dish with a diameter of 9 cm and 2 layers of filter paper. 50 full sterile sputum seeds of the same size were placed in each culture dish (Zhou *et al*., 2013). The culture dishes were then sealed with a sealing film and placed in an incubator at (25 ± 1)°C, 4000 lx, with 12 h light for 7 days. After 3 days, 6 plant seedlings were randomly taken from each culture dish and the surface moisture was gently absorbed, using filter paper. The germination potential of the alfalfa seeds was measured. After 7 days, the germination rate of alfalfa seeds, root length, seedling length, fresh weight, dry weight were measured with a ruler and one ten thousandth of a balance and the average value of each index was calculated. The calculation formulas of each index are as follows:

$$RI=(C/T-1)\times 100\%$$

The RI is the allelopathy index. Bruce *et al*. proposed that the allelopathic effect could be calculated using, the control value (C) and the treatment value (T). When RI is greater than 0 RI is expressed as a promotion. When RI is less than 0 RI is expressed as an inhibition. The absolute value of RI represents the magnitude of the allelopathic effect.

Integrated Sensitivity Effect Index (SE) represents the arithmetic mean of the RI, including germination potential, germination rate, root length, plant height, fresh weight and dry weigh (Miransari *et al*., 2014).

Bioassay of alfalfa seedling growth

According to the seed germination test, when the extracts concentration was 6 mg·mL^{-1}, the average inhibition rate of the root length was 46.7%, so the allelopathic solution concentration of the alfalfa seedling growth test was 6 mg·mL^{-1}. The alfalfa seeds were pre-germinated. 6 uniform alfalfa seedlings (in the 2-leaf stage) were then carefully transplanted into a polystyrene foam board with 6 perforations (16 cm x 9 cm) and stabilized with a tampon that was inserted into each well. The polystyrene foam board containing the seedlings was floated in a plastic pot (length 16 cm, width 9 cm, height 6 cm) containing 0.5 L of Hoagland solution and 6 mg· mL^{-1} of seed, stem and leaf, roots extract or root exudate of hairy vetch. The plastic pots were placed in an incubator at 30 ± 2°C at a light intensity of 70 µmol m^{-2} s^{-1} for 12 hours (8:00-20:00). Hoagland's solution without induction solution was used as a control. Experiments were performed in triplicate. After 7 days of treatment, all alfalfa seedlings were harvested. The root length (cm) and seedling height (cm) of the seedlings were measured with a ruler and the average value of each index was calculated.

Isolation and identification of allelochemicals

Allelochemicals in the stem and leaf extract of hairy vetch plants were isolated and identified using high performance liquid chromatography-mass spectrometry (HPLC-MS) (Sharma *et al*., 2012). The inhibitory compound was eluted at a retention time of 83-88 min. High-resolution liquid-mass acquisition data was completed by CD2.1 (Thermo Fisher). The database was searched and compared to mzCloud and mzVault (Chem Spider).

Bioassay of the Active substance

The allelopathic substance activity was determined using the experiment of seed germination and seedling growth of alfalfa under different concentrations of o-coumarin to evaluate root growth and SE.

Statistical Analysis

All experiments were carried out in triplicate. The statistical data processing was analyzed using SPSS version 16.0 using one-way ANOVA and general linear model/ univariate analyses.

RESULTS AND DISCUSSION

Effects of all extracts of the hairy vetch on seed germination of alfalfa

All extracts of hairy vetch on the seed germination potential and germination rate of alfalfa, were not significantly different ($P>0.05$) compared with the control group. When the concentration of stem and leaf extract reached 9 mg·mL^{-1}, the germination potential and germination rate were significantly lower than that of the control group. The germination potential and germination rate of each treatment group were 95% or greater (Table 1).

Effects of all extracts of hairy vetch on root length and SE during alfalfa seed germination and seedling growth

All extracts of hairy vetch had significant inhibitory effects on root length during seed germination. The inhibition of root length increased as the extract concentration increased. The effects of the stem and leaf extract were particularly remarkable. When the concentration of the stem and leaf extract reached 12 mg·mL^{-1}, the inhibition rate reached 100% (Duncan, $P<0.05$). SE of all extracts of hairy vetch on seed germination of alfalfa showed that the stem and leaf extract

Table 1: Effect of all extracts of the hairy vetch on seed germination of alfalfa. Different letters indicate significantly different values (Duncan, $P<0.05$).The same below.

Different extract	Concentration ($mg \cdot mL^{-1}$)	Seed	Stem and leaf	Root	Root exudates
Germination potential (%)	0	99.50±1.00a	99.50±1.00a	99.50±1.00a	99.50±1.00a
	3	99.00±1.15ab	98.50±1.915abc	98.00±1.64abc	95.50±2.52bc
	6	97.50±1.91abc	97.50±3.00abc	97.00±2.00abc	97.00±2.59abc
	9	96.00±3.27abc	95.50±3.00bc	97.50±1.91abc	99.00±1.15abc
	12	96.00±1.63abc	95.00±2.58c	98.50±1.00abc	97.50±1.00ab
Germination rate (%)	0	99.50±1.00a	99.50±1.00a	99.50±1.00a	99.50±1.00a
	3	99.00±1.15ab	98.50±1.92abc	98.00±1.64abc	95.50±2.52bc
	6	97.50±1.91abc	97.50±1.23abc	97.00±2.00abc	97.00±2.59abc
	9	96.00±3.27abc	95.50±3.00bc	97.50±1.91abc	99.00±1.15abc
	12	96.00±1.63abc	95.00±2.58c	98.50±1.00abc	97.50±1.00ab

Table 2: Effects of all extracts of hairy vetch on root length and SE during alfalfa seed germination.

Concentration ($mg \cdot mL^{-1}$)	Data	Seed extract	Stem and leaf extract	Root extract	Root exudates
0		2.39±0.19 a	2.39±0.19 a	2.39±0.19 a	2.39±0.19 a
3		2.57±0.23 a	1.40±0.12 cd	1.34±0.19 d	2.14±0.15 b
6	Root length	1.60±0.28 c	0.67±0.10 gh	0.98±0.14 f	1.23±0.19 de
9	(cm)	1.40±0.44 cd	0.23±0.56 i	0.50±0.13 h	1.10±0.27 ef
12		0.74±0.21 g	0±0 i	0.12±0.50 i	0.87±0.09 fg
3		-12.40±0.51 a	-10.50±1.32 a	-10.52±2.75 a	-14.43±3.11 ab
6	SE (%)	-21.76±1.84 c	-19.25±3.54 c	-18.60±2.49 bc	-11.25±2.76 a
9		-29.11±4.00 de	-28.40±3.83 d	-23.48±1.88 c	-22.07±1.36 c
12		-33.71±4.33 e	-39.07±5.51 f	-33.79±4.51 e	-30.29±3.37 de

had the greatest significant inhibitory effect on the germination process at a concentration of 12 $mg \cdot mL^{-1}$, with an inhibitory effect reaching 39.18±9.12% (Duncan, $P<0.05$) (Table 2). The root exudates of the hairy vetch had no significant inhibitory effect on the root length, but the seed, stem and leaf and root extracts had significant inhibitory effects, especially the stem and leaf extract during alfalfa seedling growth. When the seedling was treated with the stem and leaf extract, the alfalfa root length was only 2.72±1.16 cm, compared to a root length of 10.18±1.83 cm in the control condition. The stem and leaf extract had the significant inhibitory effect on the growth of the alfalfa seedling (52.91±3.99%). These findings show that the allelopathic effect of the stem and leaf extract on alfalfa was stronger than the effects of the seed, root extract, or the root exudates of the hairy vetch. In summary, hairy vetch is a kind of crop with strong allelopathic effect. And the test results show that there were more naturally occurring allelochemicals in the stem and leaf of the hairy vetch plant than in the other parts of the plant (Table 3).

Comprehensive allelopathy index analysis revealed that the allelopathic effects of extracts from different parts of the hairy vetch on alfalfa were different and its allelopathic intensity was as follows: stem and leaf extract> root extract ≥ seed extract> root exudates, indicating that the important part of the allelochemicals in the hairy vetch is the stem and leaf.

Isolation and identification of main allelochemicals in the stem and leaf extract of hairy vetch

A total of 479 allelochemicals were identified (Table S1). O-coumaric acid is the main allelochemical identified in the stem and leaf of hairy vetch. The degree of o-coumaric acid in mzCloud and mzVault was found to fully match, indicating that o-coumaric acid is contained in the stem and leaves of the hairy vetch plant (Table 4). O-coumaric acid is a white needle crystal with a melting point of 214°C that is easily soluble in alcohol and hot water (Fig 1A). The chromatogram of the compound appeared at a retention time of about 6.834 minutes (Fig 1B).

Evaluation of the allelopathic effect of o-coumaric acid on alfalfa

Bioassays were used to evaluate the effect of o-coumaric acid on root growth and SE (Table 5). The results showed that the root length was completely inhibited at a substance concentration of 1.6 $mg \cdot mL^{-1}$ during seed germination, the root length was completely inhibited at a concentration of 12 $mg \cdot mL^{-1}$ with stem and leaf extract (Table 2). Therefore, we conclude that o-coumaric acid has a stronger allelopathic effect than the extract of the stem and leaf extract of the hairy vetch on alfalfa roots during germination. The alfalfa root length was only 1.54±0.39 cm at substance concentration of 2.5 $mg \cdot mL^{-1}$, compared to a root length of 15.16±1.69 cm in the control condition. The o-coumaric acid

Table 3: Effects of all extracts of hairy vetch on root length and SE during alfalfa seedling growth.

Concentration (mg·mL^{-1})	Data	Seed extract	Stem and leaf extract	Root extract	Root exudates
0	Root length (cm)	10.18±1.83 a	10.18±1.83 a	10.18±1.83 a	10.18±1.83 a
6		6.68±0.45 b	2.72±1.16 c	5.99±0.97 b	10.44±1.41 a
6	SE (%)	-9.79±3.67 a	-52.91±4.00 c	-28.74±0.67 b	-6.61±2.65 a

Table 4: The matching degree of O-coumaric acid in mzCloud and mzVault and its peak area.

Name	Formula	mzCloud Search	mzVault Search	mzCloud Best Match	mzVault Best Match	Area (Max.)
o-coumaric acid	C9H8O3	Full match	Full match	84.5	70.3	12773964.35

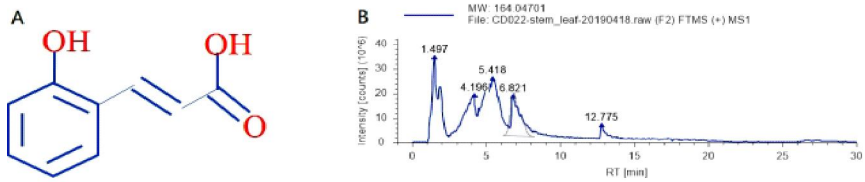


Fig 1: The structure and chromatogram of O-coumaric acid. A The structure of O-coumaric acid. B The chromatogram of o-coumaric acid was confirmed using HPLC-MS.

Table 5: Evaluation of the allelopathic effect of O-coumaric acid on root length and SE during alfalfa seed germination and seedling growth.

Seed germination			Seedling growth		
O-coumaric acid concentration (mg·mL^{-1})	Root length (cm)	SE (%)	O-coumaric acid concentration (mg·mL^{-1})	Root length (cm)	SE (%)
0	2.40±0.22 a	-11.51±3.29 a	0	15.16±.1.69 a	
0.05	1.54±0.23 b	-21.09±4.32 b	0.5	7.73±0.61 b	-56.62±6.04 a
0.1	0.94±0.15 c	-16.19±2.10 ab	1	7.95±1.02 b	-60.63±6.45 bc
0.2	0.91±0.13 c	-24.46±3.66 bc	1.5	3.79±0.34 c	-65.24±4.17 ab
0.4	0.64±0.16 d	-36.89±4.56 c	2	2.62±0.49 d	-69.55±4.17 bc
0.8	0.42±0.09 e	-38.62±3.95 c	2.5	1.54±0.39 d	-78.90±5.60 c
1.6	0±0 f	-38.62±3.41 c			
3.2	0±0 f	-41.07±3.01 d			

had the significant inhibitory effect on the growth of the alfalfa during seedling growth. The reason for the high content of allelochemicals in stem and leaf may be that the leaching and volatilization of allelopathic substances in stems and leaves is one of the main modes of action of allelochemicals. In the process of leaching and volatilization, many substances are wasted, so in order to achieve the effect, the stem and leaf must release more allelochemicals. O-coumaric acid is a phenolic compound and Zhong-Yu Zhou *et al* have shown that phenolic compounds such as 3-(2-hydroxyphenyl) propyl methyl malonate, 3-(2-hydroxyphenyl) -1-propanol and o-coumaric acid remarkably showed inhibition activity against Arabidopsis seed germination at a concentration of 1.0 mM. And o-coumaric acid further found to show obvious inhibitory activity on retarding the seedling growth of Ar. thaliana cultured in soil medium (Quan *et al*., 2019).

O-coumaric acid is moderately soluble in water. If released in water or soil, it is expected to bind to soil particles or suspended particles (Sen *et al*., 2013). The present results indicate that the isolated compound inhibited root growth of alfalfa. The mechanism of inhibition may be due to changes in plant cell structure, compromises the lipidome, membrane integrity and photosynthesis (Poulin *et al.*, 2018). The imbalance of the antioxidant system, the destruction of the activity and function of various enzymes, the influence of nutrient absorption on the roots of plants or the effects on nucleic acids and nucleic acids (Pandya *et al*., 2017), destroys the balance of hormones in the seeds. The isolated compound from the plant material, O-coumaric acid, may contribute to the allelopathic effects of hairy vetch.

CONCLUSION

The allelopathic effects of four methanol extracts of the hairy vetch on seed germination and seedling growth of alfalfa were studied to evaluate the allelopathic effects, we concluded that the allelopathic effect of the stem and leaf extract was the strongest. Next, isolated and identified 479 allelochemicals from the stem and leaf extract of hairy vetch using HPLC-MS. Finally, the allelochemicals of o-coumaric

acid were screened out and may contribute to the allelopathic effects of hairy vetch.

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