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Identification of phenolic acids in rhizosphere soil of continuous cropping of *Salvia miltiorrhiza* Bge

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ABSTRACT

In this study we used, ultra high-performance liquid chromatography-quadruple time-of-flight-mass spectrometry to isolate and identify the allelochemicals in the rhizosphere soil of *S. miltiorrhiza* Bge. Five phenolic compounds (Benzoic acid, vanillin, ferulic acid, vanillic acid, and *p*-hydroxycinnamic acid) were isolated from the rhizosphere soil. The *p*-hydroxycinnamic acid content was maximum in the rhizosphere soil of *S. miltiorrhiza* Bge. Bioassays showed that seed germination of *S. miltiorrhiza* Bge was significantly inhibited by *p*-hydroxycinnamic acid at 38.51 $\mu g \cdot mL^{-1}$ concentration and the inhibition increased with the increase in *p*-hydroxycinnamic acid concentration i.e. concentration dependent. The seeds germination was completely inhibited at 154.04 $\mu g \cdot mL^{-1}$ concentration. In addition, the applied *p*-hydroxycinnamic acid decreased the seedlings height and growth of rhizomes of *S. miltiorrhiza* Bge. This study indicated that *p*-hydroxycinnamic acid may be an important autotoxin of *S. miltiorrhiza* Bge.

Key words: Autotoxins, bioassay, continuous cropping, HPLC, phenolic acids, rhizosphere soil, *Salvia miltiorrhiza* Bge, seed germination, seedling growth, UPLC-Q-TOF-MS^n

INTRODUCTION

Soil sickness refers to the abnormal growth and development of crops due to the continuous cultivation of the same crop on the same land (4,13,17). Plants produce secondary metabolites (organic acids, phenolic acids, and saponins) as by-products and release them in the surrounding environment (11,22). The excessive accumulation of metabolites may lead to self-poisoning of soil, e.g. inhibiting the seeds germination and growth of same plant and related species. Secondary metabolites are also referred as autotoxins and often related with soil sickness of crops (13,22). The continuous mono-cropping of same crop on the same land causes the accumulation of secondary metabolites (8). Soil sickness has become the focus of research in the field of medicinal plants cultivation and has been reported in several medicinal plants [*Panax ginseng* C.A. Mey (8), *Panax quinquefolium* L. (18), *Panax notoginseng* (21) and *Rehmannia glutinosa* (Gaetn.) Libosch. ex Fisch. et Mey. (9)] cropping systems. Li *et al.* (9) identified 5 phenolic acids [gallic acid (GA), salicylic acid (SA), 3-phenylpropionic acid (3-PA), benzoic acid (BA), and cinnamic

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acid (CA)] in ginseng rhizosphere soil. Amongst these, the GA, SA, 3-PA and BA significantly increased the disease severity of *Cylindrocarpon* root rot of ginseng and decreased the plant growth. It seems that perhaps, phenolic acids are one of the main allelochemicals that cause soil related diseases in medicinal plants.

Salvia miltiorrhiza Bge (family Labiatae) is medicinal plant. The soil sickness caused by its continuous cultivation has reduced its yield and the synthesis of its main medicinal components (Salvianolic acid B and tanshinones). The continuous cultivation of *S. miltiorrhiza* Bge induces soil sickness due to release and accumulation of allelochemical in soil (**7**). Previous studies have shown that phenolic acids, as allelochemicals, inhibits or delays the germination and growth of medicinal plants (8,9). Therefore, phenolic acids might be one of the main allelochemicals that cause soil sickness in *S. miltiorrhiza* Bge.

This study aimed to (i). identify the phenolic acids in the rhizosphere soil of *S. miltiorrhiza* (ii). verify and quantify the main phenolic acids in the rhizosphere soil of *S. miltiorrhiza* after continuous monocropping and (iii). to investigate the effects of detected allelochemicals on the seed germination and seedling growth of *S. miltiorrhiza*.

MATERIALS AND METHODS

Soil sampling

The soil samples were collected from the *S. miltiorrhiza* Bge plantation base in Shangluo, Shanxi Province, China (108°34′20″-111°1′25″East longitude and 33°2′30″-34°24′40″North latitude, mean annual temp: 7.8-13.9 ºC, mean annual precipitation: 696.8-830.1 mm and mean annual sunshine hours: 1848.1-2055.8 h). The test soil was collected from the continuous cropping area of *S. miltiorrhiza* Bge from randomly selected plots (2 m × 2 m) each. The whole *S. miltiorrhiza* Bge plants were harvested and their rhizosphere soil was collected by shaking the roots and mixed the soil samples (9). The control soil was from the area not planted with *S. miltiorrhiza* Bge in last 5-years. The soil samples were dried naturally at room temperature (~25 ºC). The experiments were conducted at Zhejiang Sci-Tech University, Zhejiang.

Phenolic acids in rhizosphere Soil

Extract preparation: The soil extracts were prepared as per slightly modified method of Lee *et al*. (2). The dried soil samples were, crushed and passed through 60-mesh sieve. Soil (25 g) was added to 150 mL 2 mol·L^{-1} NaOH solution and shaken for 24 h. Then the samples were centrifuged at 8000 r·min^{-1} for 20 min at 25 ºC. The supernatant was acidified with 5 mol·L^{-1} hydrochloric acid to adjust the pH to 2.5 and kept in dark for 2 h. The mixture was centrifuged again and the supernatant was extracted with ethyl acetate. The extracts were dried at 40 °C. Then 5 mL of 80 % methanol was added to dissolve the dried mass, filtered and stored at -20 °C. The following lab bioassays were conducted to determine the effects of phenolic acids and potential autotoxins in *S. miltiorrhiza* seed germination.

I. UPLC-Q-TOF-MS^n and HPLC Analysis Conditions

(i). UPLC-Q-TOF-MS^n Conditions

Positive ion mode detection conditions:

Mass spectrometry conditions: Ion source: capillary voltage: 3.01kV; carrier gas: ordinary nitrogen; carrier temperature: 500 °C; carrier gas velocity: 12 L·min^{-1}; scan range: m/z = 100-000.

Chromatographic conditions: Agilent Infinity Lab Poroshell 120EC-C18 column, column temperature: 40 °C, mobile phase: 0.1 % methanol (A) and acetonitrile (B), flow rate: 0.3 mL·min^{-1}, gradient elution procedure: 0:00-1:50 min, 95 %-90 % A; 1:50-2: 85 min, 90 %-80 % A; 2:85-6:50 min, 80 %-75 % A; 6:50-8:65 min, 75 %-70 % A; 8:65-11:50 min, 70 %-65 % A; 11:50-14:50 min, 65 %-5 % A; 14:50-17:00 min, 5 %-95 % A.

Negative ion mode detection conditions: Mass spectrometry and chromatographic conditions were the same as described in positive ion mode detection conditions.

Gradient elution procedure: 0:00-1:50 min, 95 %-90 % A; 1:50-2:85 min, 90 %-80 % A; 2:85-6:50 min, 80 %-75 % A; 6:50-8:65 min,75 %-70 % A; 8:65-10:00 min,70 %-95 % A.

(ii). HPLC Conditions

Waters Sunfire C18 column; column temperature: 30 °C; injection volume: 30 μL; mobile phase: 0.02 % phosphoric acid water (A) and acetonitrile (B); detection wavelength: 270 nm (vanillin, *p*-hydroxycinnamic acid), 240 nm (benzoic acid), 290 nm (vanillic acid), and 320 nm (ferulic acid), flow rate: 1.0 mL·min^{-1}. Gradient elution procedure: 0:00-10:00 min, 95 %-90 % A; 10:00-20:00 min, 90 %-80 % A; 20:00-40:00 min, 80 %-75 % A; 40:00-42:00 min, 75 %-95 % A.

Preparation of standard solution and preparation of standard curve: Standard solutions of vanillin, benzoic acid, *p*-hydroxycinnamic acid, caffeic acid, ferulic acid and, vanillic acid with concentrations of 0.279, 0.294, 0.269, 0.100, 0.100, and 0.100 mg·mL^{-1}, respectively, were prepared and stored at 4 °C protected from light. The standard curve was prepared by HPLC. Standard curves for 1, 2, 5, 10, 15, and 20 μL of standard solution were accurately drawn, in which the injection volume (μg) was the abscissa and the standard peak area (A) was the ordinate. The gradient elution procedure was referenced in HPLC conditions.

II. Laboratory Bioassay

The petriplate bioassay was done to determine the effects of different concentrations of three phenolic acids solutions on the germination and seedlings growth of *S. miltiorrhiza*. The experimental treatments consisted of 2 factors: (i). 3- phenolic acids (Benzoic acid, vanillin, and *p*-hydroxycinnamic acid) and (ii). their concentrations 5 (each phenolic acid has different concentration depending on its content in rhizosphere soil of *S. miltiorrhiza*) shown in Table 1. The treatments were replicated thrice in Complete Randomized Design. The sterilized seeds were sown in the petri dishes and treated with phenolic acids solutions. The germination was recorded for 7 days after sowing.

Table 1. Exogenous phenolic acid concentrations (μg·mL^{-1}) used in petri plate bioassay.

Phenolic acid	Phenolic acid concentrations (μg·mL^{-1})					
	CK (Control)	**A**	**B**	**C**	**D**	**E**
Benzoic acid	0	1.09	2.18	4.36	8.72	17.44
Vanillin	0	7.21	14.42	28.84	57.68	115.36
p-Hydroxycinnamate acid	0	9.6275	19.255	38.51	77.02	154.04

Seed germination: It was determined as per Yang Ming's method (20). The *S. miltiorrhiza* seeds were sterilized with 0.5 % NaClO solution for 20 min and then rinsed 5 times with sterilized distilled water. The seeds were soaked in 50 mL sterilized distilled water for 60 min. Twenty sterilized seeds were placed in 9 cm dia petridish lined with two sterilized filter papers and 10 mL treatment solution of corresponding concentration was added in each petri dish. In control sterilized distilled water was used. Subsequently 1 mL of treatment solution was added daily to maintain moisture in petri dishes. Five concentrations of each phenolic acid (Benzoic acid, vanillin, and p-hydroxycinnamate acid) were used (Table 1). Seeds were cultured in incubator (12 h :12 h light: dark cycles and 60 % relative humidity, 25 °C). The number of germinated seeds was recorded daily for 7 days and germination was calculated as under (6):

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated seeds}}{20} \times 100\%$$

Statistical analysis

Statistical analyses were performed with SPSS statistical software 22.0 (USA). One-way ANOVA followed by Tukey's HSD post hoc test was used for statistical analysis. The P values < 0.05 and < 0.01 were considered statistically significant and highly statistically significant, respectively.

RESULTS AND DISCUSSIONS

UPLC-Q-TOF-MS^n Analysis of Phenolic Acids in *S. miltiorrhiza* Rhizosphere Soil

(i). Ferulic acid, *p*-hydroxycinnamic acid and vanillic acid: Three phenolic acids (Ferulic acid, vanillic acid and *p*-hydroxycinnamic acid), were identified in the negative ion mode (Table 2). The two characteristic fragment ions, m/z 193.0500 and 134.0372 of ferulic acid were obtained through secondary mass spectrometry, and m/z 134.0372 was attained after $[M-H]^-$(m/z 193.0500) lost CO_2 and CH_3. The specific cracking rule of vanillic acid is shown in Figure 1. $[M-H]^-$(m/z 167.0372) cracked and lost one CH_3 to produce m/z 152.0106. $[M-H-CH_3]^-$(m/z152.0106) further cracked to release CO_2 leading to the formation of m/z 108.0202. According to the second-stage mass spectrometry of *p*-hydroxycinnamic acid, two characteristic fragment ions, m/z 163.0427 and 119.0501, were obtained, wherein m/z 163.0427 is $[M-H]^-$ and m/z 119.0501 is $[M-H]^-$(m/z 163.0427) after losing a CO_2 (Fig.1).

Table 2. Phenolic acids in soil extracts identified by UPLC-Q-TOF-MS^n in negative ion mode.

Phenolic acid	RT (min)	$[M-H]^-$(m/z)	Debris peak (m/z)	Reference
Ferulic Acid	5.59	193.0500	134.0372	134.2[(1)]
p-Hydroxycinnamic acid	5.14	163.0427	119.0501	119.2[(1)]
Vanillin acid	4.12	167.0372	152.0106, 108.0202	151.6[(11)], 108.0[(1)]

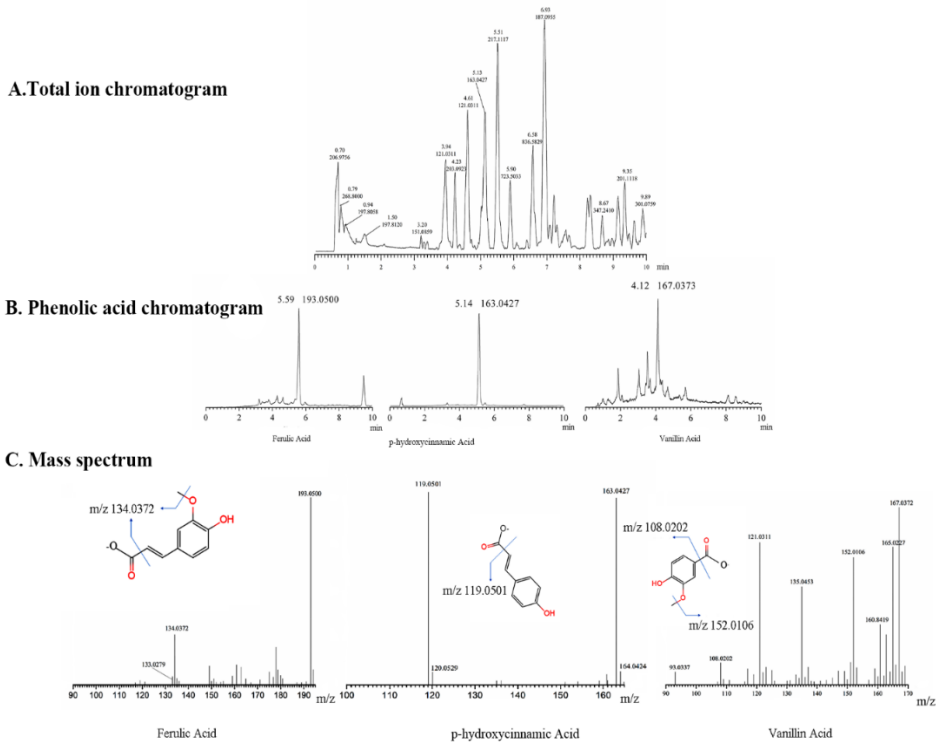

Figure 1. UPLC-Q-TOF-MS^n separation of *p*-hydroxycinnamic acid, ferulic acid, and vanillic acid in negative ion mode: A: Total ion chromatogram (TIC), B: Phenolic acid chromatogram; C: Mass spectrum.

(ii). Benzoic acid, vanillin and vanillic acid: Three phenolic acids (Benzoic acid, vanillin and vanillic acid), were identified in the positive ion mode (Table 3). According to the secondary mass spectrometry of benzoic acid, $[M+H]^+$ (m/z 123.0466) one CO molecule was removed to obtain the fragment peak m/z 95.0501, that is, $[M+H-CO]^+$=m/z 95.0501. In Vanillic acid, two fragment peaks in positive ion mode (m/z 169.0480 and 151.0399) were obtained, by removal of one H_2O molecule and $[M+H-H_2O]^+$ split the fragment peak m/z 151.0399 (Fig. 2). The results of UPLC-Q-TOF-MS^n analysis in this experiment were consistent with previous experimental results (1); thus, the results were accurate and reliable (Tables 2 and 3).

Table 3. Phenolic acids in soil extracts identified by UPLC-Q-TOF-MS^n in positive ion mode.

Phenolic acid	RT (min)	$[M+H]^+$(m/z)	Debris peak (m/z)	Reference
Benzoic acid	4.56	123.0466	95.0501	95.0[3]
Vanillin	3.09	153.0553	138.0570	138.0[3]
Vanillin acid	1.86	169.0480	151.0377	151.0[14]

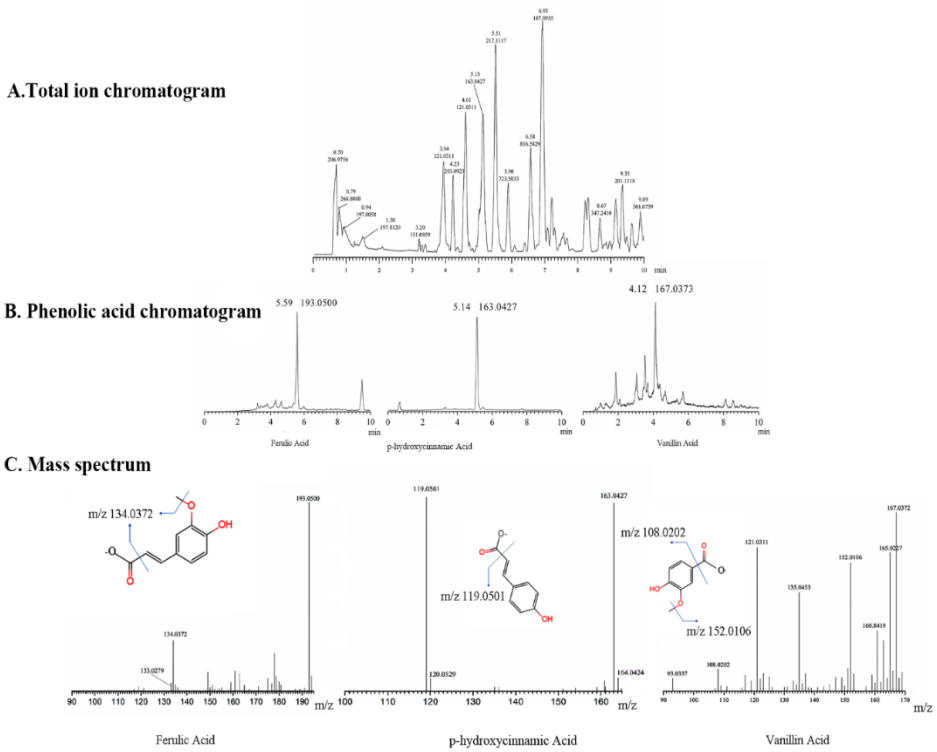

Figure 2. UPLC-Q-TOF-MS^n separation of benzoic acid, vanillin and vanillic acid in positive ion mode: A is the total ion chromatogram; B is the phenolic acid chromatogram; C is the mass spectrum.

Allelochemicals in Rhizosphere Soil of *S. miltiorrhiza*

There are some differences in the allelochemicals produced by medicinal plants. In *Rehmannia*, the vanillic acid, *p*-hydroxybenzoic acid, syringic acid and ferulic acid work together to cause soil sickness (9). *P. notoginseng* is more sensitive to the allelopathic effects of ferulic acid and *p*-hydroxybenzoic acid (21). This study detected and separated 5-allelochemicals (Benzoic acid, vanillin, ferulic acid, vanillic acid and p-hydroxycinnamic acid) by UPLC-Q-TOF-MS^n by HPLC from the rhizosphere soil of *S. miltiorrhiza* Bge. Figure 3 showed that the peak time of these 5-phenolic acids in the soil extract was highly consistent with the peak time of the corresponding components in the standard. This confirmed that soil used in the continuous cultivation of *S. miltiorrhiza* contained 5-phenolic substances: ferulic acid, vanillin, vanillic acid, benzoic acid and *p*-hydroxycinnamic acid.

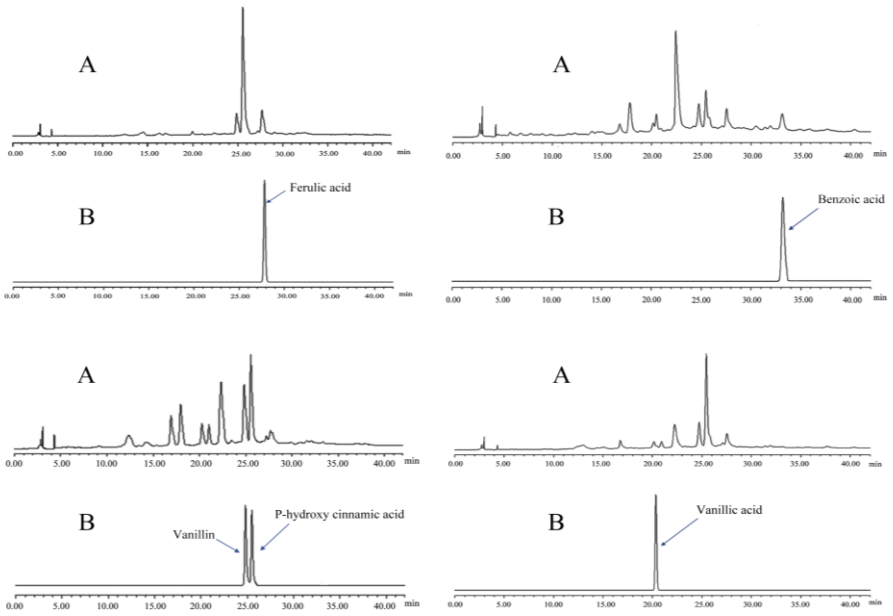

Figure 3. Benzoic acid, ferulic acid, vanillic acid, vanillin and *p*-hydroxybenzoic acididentified by HPLC: A : Soil sample phenolic acid, B : Standard phenolic acid.

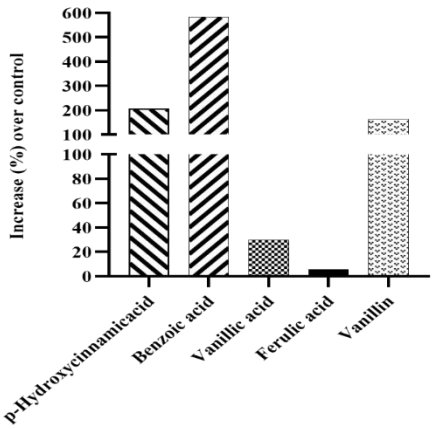

Figure 4. Increase (%) of phenolic acids contents in rhizosphere soils after 2-years continuous planting of *S. miltiorrhiza* over control.

HPLC quantitative analysis showed that the contents of 5-phenolic acids in the rhizosphere soil of 5-years continuous cropped soil was increased over control soil (not

planted with *S. miltiorrhiza* Bge) (Fig. 4). The contents of benzoic acid, *p*-Hydroxycinnamic acid and vanillin greatly increased in *S. miltiorrhiza* grown soil by 583.61 %, 207.21 % and 163.08 % respectively, followed by vanillic acid (30.04 %), and ferulic acid (5.67 %). Therefore, benzoic acid, *p*-hydroxycinnamic acid, and vanillin were selected for the germination bioassay of *S. miltiorrhiza* Bge seeds.

Petri plate bioassay

(i). *p*-Hydroxycinnamic acid: The *p*-hydroxycinnamic acid at lower concentration (38.51 $\mu g \cdot mL^{-1}$) significantly inhibited the seed germination of *S. miltiorrhiza* Bge. The inhibitory effects increased with the increase in *p*-hydroxycinnamic acid concentration i.e. concentration dependent. The seeds did not germinate at concentration of 154.04 $\mu g \cdot mL^{-1}$. In addition, the study found that the growth of shoot and underground parts of *S. miltiorrhiza* seedlings were significantly inhibited by *p*-hydroxycinnamic acid at concentration >38.51 $\mu g \cdot mL^{-1}$. As the *p*-hydroxycinnamic acid concentration increases, the rhizomes of *S. miltiorrhiza* seedlings become smaller and less branches. The seed germination and seedling growth varied in response to different treatments (Fig. 5). The medicinal value of *S. miltiorrhiza* is in its rhizomes and as its effective medicinal components: tanshinones and salvianolic acid are accumulated in the rhizomes (16). The salvianolic acid is synthesized by two metabolic pathways: (i). Phenylpropane metabolism pathway and (ii). rosmarinic acid metabolism pathway (19). Cinnamic acid produces the 4-coumaric acid under the action of cinnamic acid 4-hydroxylase, and 4-coumaroyl-CoA was obtained by the 4-coumaric coenzyme A. The 4-Hydroxyphenyllactate was produced by tyrosine pathway and 4-coumarinyl coenzyme A, produces phenolic acids (rosmarinic acid and salvianolic acid B), under the action of rosemary acid synthase and cytochrome P450 (cyp98a14). The biosynthetic pathways of tanshinones includes the mevalonate pathway in the cytoplasm and the non-mevalonate pathway in the plastid (10). Therefore, it is possible that the high concentration of *p*-hydroxycinnamic acid inhibits the key enzyme activity involved in biosynthetic pathways of salvianolic acid and tanshinones, even decreasing the growth and development of *S. miltiorrhiza* and ultimately declining the medicinal quality. In addition, *p*-hydroxycinnamic acid also inhibits the phosphorylase enzymes required for seed germination (1) along with restraining the synthesis of some key enzyme intermediate products (2), reducing the seeds germination.

(ii). Vanillin: Its inhibitory effect was not concentration-dependent. Its low doses 7.21, 4.42, and 28.84 $\mu g \cdot mL^{-1}$ did not significantly affect the seed germination of *S. miltiorrhiza*. However, the vanillin concentration of 57.68 $\mu g \cdot mL^{-1}$ inhibited the seed germination of *S. miltiorrhiza* and 115.36 $\mu g \cdot mL^{-1}$concentration drastically reduced the seed germination. Thus higher vanillin concentrations significantly inhibited the seed germination and the root number of *S. miltiorrhiza* seedlings (Fig. 5).

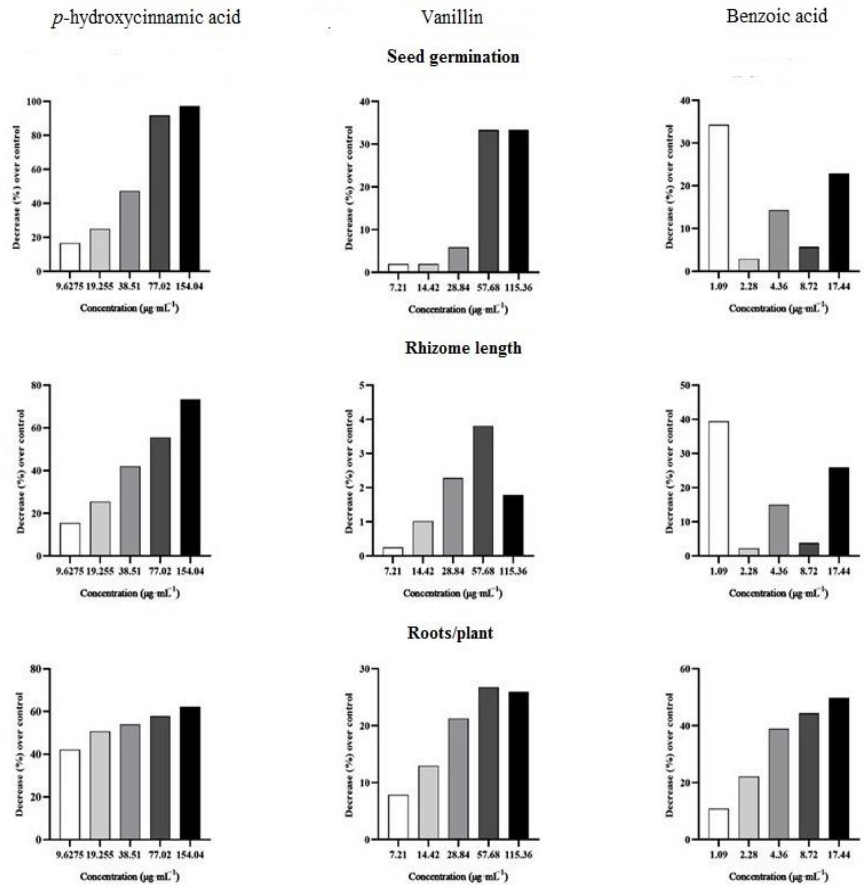

Figure 5. Effects of phenolic acids application on seed germination and seedlings growth of *S. miltiorrhiza.*

(A). The inhibition rate of seed germination of *S. miltiorrhiza* after treatment with *p*-hydroxycinnamic acid, vanillin and benzoic acid than control (0 μg/mL). (B). The decrease (%) of rhizome length of *S. miltiorrhiza* after treatment with *p*-hydroxycinnamic acid, vanillin and benzoic acid than control (0 μg/mL). (C). The decrease (%) of roots/plant of *S. miltiorrhiza* after treatment with *p*-hydroxycinnamic acid, vanillin and benzoic acid than control (0 μg/mL).

(iii). Benzoic acid: The inhibitory effects of various concentrations of benzoic acid solution on the seed germination of *S. miltiorrhiza* were not prominent. The seedlings germinated with many rhizomes and branches, regardless of the benzoic acid solution concentration (Fig.5). However, with increasing concentration of benzoic acid solution, the seedling leaves gradually turned yellow and leaf area was decreased. Its low concentration (17.44 $\mu g \cdot mL^{-1}$) stimulated the growth of rhizomes of *S. miltiorrhiza* but inhibited the leaves development (Fig. 6).

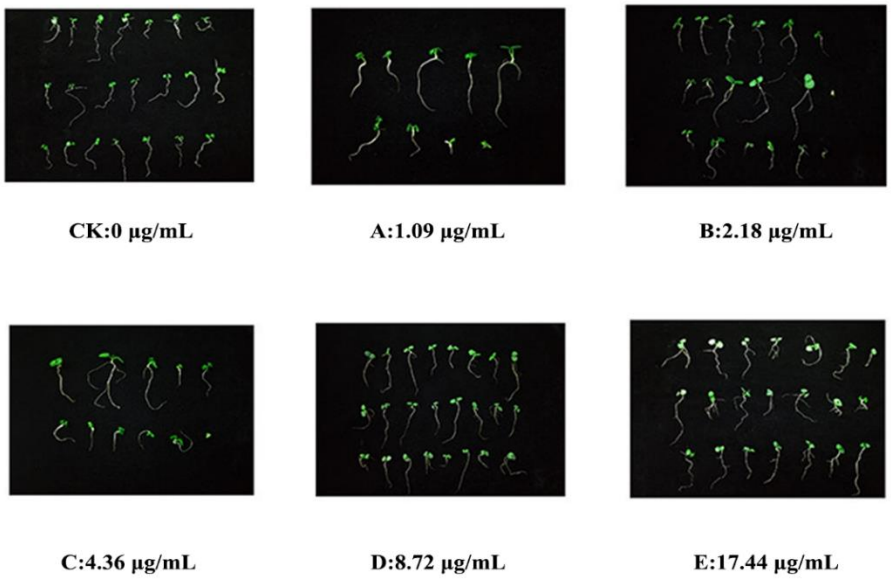

Figure 6. Effects of benzoic acid solutions on the seedlings growth of *S. miltiorrhiza.*

Therefore, we hypothesize that *p*-hydroxycinnamic acid might be a potential allelochemical that affects the seed germination of *S. miltiorrhiz*a and the inhibitory rate amplified with the increase of allelochemical concentration i.e. concentration dependent. However, the benzoic acid and vanillin did not inhibit the seeds germination of *S. miltiorrhiza*.

CONCLUSIONS

In the soil continuously cropped with *S. miltiorrhiza* for 2 years, the content of *p*-hydroxycinnamic acid increased than control soil (where *S. miltiorrhiza* was not planted in last 5-years). Bioassays showed that the inhibitory effects of *p*-hydroxycinnamic on the germination and seedling growth of *S. miltiorrhiza* increased as its concentration increased i.e. concentration dependent. Therefore, the study reflects that *p*-hydroxycinnamic acid may be an important autotoxin that causes the soil sickness of *S. miltiorrhiza*.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest : The Authors declare no conflict of Interest.

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PUBLISHER NOTE