https://doi.org/10.26651/allelo.j/2019-47-2-1238 0971-4693/94 Euro 20.00
Allelopathy Journal 47 (2): 283-294 (July, 2019) *International Allelopathy Foundation* 2019
Table: 3, Figs : 9, Photograph: 1

Allelopathic effects of *Juglans regia* L. leaf extracts on seedling growth of *Isatis tinctoria* L.

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(Received in revised form: May 07, 2019)

ABSTRACT

We studied the allelopathic effects of aqueous leaf extracts from *Juglans regia* L. on growth (seedling growth and biomass) and physiological parameters (photosynthesis, chlorophyll, soluble protein, soluble sugar, proline, malondialdehyde content) and enzymatic activities (peroxidase, superoxide dismutase and catalase enzymes) of *Isatis tinctoria*. The lowest concentration of e aqueous extract (0.005 g/mL) stimulated the seedling growth and biomass accumulation. All concentrations assayed (0.005 g/mL, 0.05 g/mL and 0.1 g/mL) inhibited the respiration rate, net photosynthesis rate, stomatal conductance and chlorophyll content of *Isatis tinctoria* seedlings. However, they also increased the intercellular CO_2 of *Isatis tinctoria* seedlings. The contents of soluble protein, soluble sugar, proline, malondialdehyde content and the activities of the peroxidase, superoxide dismutase and catalase enzymes varied with leaf extract concentrations.

Key word: Allelopathic effects, aqueous extracts, *Isatis tinctoria*, *Juglans regia* L., leaf, physiological responses, seedling growth.

INTRODUCTION

Allelopathic effects are widespread in agroforestry ecosystems, affecting the structure of community, succession and component crop yield (1,22,34,35). The problem of soil sickness in continuous cropping, forest regeneration and biological invasion are related to allelopathy (2,24,30,36). The studies of inter-specific relationships in agroforestry system helps us to understand the stability of ecosystem structure and function, and provides theoretical guidance to explore the rational and efficient use of resources and selection of suitable tree species for agroforestry systems (25). The mass concentration effect of allelochemicals of donor plants is an important aspect of allelopathy. The allelochemicals concentration in donor plants affects the biological activity and biomass of receptor plants (31).

The allelopathic potential of > 100 plants have been investigated, but, there are few reports on forest allelopathy (6,11,15,20,38,40). *Juglans regia* L. is major forest specie in China and has allelopathic effects on adjacent plants (9,21,27). Its major allelochemical

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'juglone' affects the seed germination, seedling growth, photosynthesis, respiration and chlorophyll content of neighbouring plants (8,14,17,37,39). Its leaves contains the phenolic acids and flavonoids (28). Owing to *Juglans regia* L. high productivity, fast growth and adaptability to management, it is most suitable tree for intercropping in agroforestry systems (29). Hence, it has been planted in Agroforestry systems in large areas in the Loess Area of Northern Wei River in Shaanxi (5). However, its allelopathic effects in intercropping with Chinese herbal medicinal plants is not known. Hence, this study aimed to evaluate the effect of aqueous leaf extracts of *Juglans regia* L. on growth and physiological responses of *Isatis tinctoria* (Medicinal plant that could be intercropped with *Juglans regia* L.).

Isatis tinctoria seeds

Isatis tinctoria seedlings

Isatis tinctoria pot culture

MATERIALS AND METHODS

Leaf aqueous extracts: Fresh leaves of *Juglans regia* L. were collected in May 2008, from the Nursery Garden, College of Forestry at Northwest A&F University, Yangling, Shaanxi, China. (East longitude 108°4′3″, north latitude 34°15′43″, altitude : 460 m, annual average temperature :12.9 ℃, annual average rainfall : 635.1 mm). The fresh leaves were cut into 1 cm pieces of and 10 g cut material were immersed in 100 ml-distilled water for 24 h at room temperature (20-25 ºC). The aqueous extract (0.1 g/mL) was filtered through 3-layers of filter paper. For the pot experiment, the extract was further diluted to 0.005 g/mL and 0.05 g/mL.

Isatis tinctoria seeds were purchased from the Medicinal market of Xi'an, Shaanxi, China. The seeds were surface-sterilized with 10:1 water/bleach (commercial NaOCl) solution for 5 mins, and washed six times with distilled water. Then, seeds were sown in the field and after 15- days, the seedlings of *Isatis tinctoria* were available for transplanting in pots.

Pot culture: Plastic pots (20 cm dia, 27 cm depth) were filled with 10 kg loess soil（pH: 7.65, Organic matter: 4.45%, Alkali-hydrolyzable N: 116 $mg \cdot kg^{-1}$, Available P: 19.8 $mg \cdot kg^{-1}$, Available K: 64.5 $mg \cdot kg^{-1}$.) Five seedlings of *I. tinctoria* were transplanted per pot. Each pot was irrigated at 10 and 30 days after Transplanting times, the experiment duration was 40-days. Each irrigation consisted of 100 mL fresh leaf aqueous extracts concentrations of (0.005 g/mL, 0.05 g/mL and 0.1 g/mL) or water (Control) as per treatment. Treatments were replicated thrice. The seedling height and stem girth (caliper) of *Isatis tinctoria* were measured at 10, 20, 30 and 40 day after transplanting. Forty days after transplanting, the plants were uprooted and their fresh weight, dry weight, aboveground and underground biomass of each plant were measured.

Photosynthetic indexes and Chlorophyll : A portable photosynthesis system (Li-6400; LI-COR Inc., Lincoln, NE, USA) was used to determine the leaf transpiration rate, net photosynthesis rate, stomatal conductance and intercellular CO_2 concentration of *Isatis tinctoria.* A portable chlorophyll content meter (CCM-200, OPTI-science, USA) was used to determine the chlorophyll contents of *Isatis tinctoria*.

Physiological indexes: Leaves of *Isatis tinctoria* were collected at 10,20,30 and 40 day after transplanting, and stored in refrigerator for one month. The frozen leaves were used to determine the soluble proteins with Coomassie blue staining (41), soluble sugars by anthrone colorimetry (41), proline by the acid ninhydrin method (34), malondialdehyde (MDA) by the thiobarbituric acid method (41), the superoxide dismutase activity (SOD) by the assay system consisting in methionine, riboflavin and NBT (12), and the peroxidase (POD) and catalase (CAT) activities by a direct spectrophotometric assay (4).

Response Index: The Williamson's response indices (*RI*) were used as under (32):

$$T \geq C,\ RI = 1 - C/T, \text{ and } T \leq C,\ RI = T/C - 1,$$

Where, *RI:* Allelopathic effects, C: Control data, T: Treatment data

Statistical analysis: All data were analyzed statistically using one-way ANOVA test in the software SPSS 16.0. Differences among means were detected with Duncan's multiple range test at 5% level.

RESULTS AND DISCUSSION

Seedling growth

The *Juglans regia* L. leaf extracts at different concentrations influenced the stem length, root collar diameter and seedling biomass (Fig. 1, 2 and Table 1). The *Juglans regia* L. leaf aqueous extracts at lower concentration (0.005 g/mL) stimulated the stem length of *Isatis tinctoria*. After 40 days at 0.005 g/mL concentration, the stem length reached 12.35 cm. However, higher concentration of leaf extracts (0.1 g/mL) inhibited the stem length (10.97 cm at 40^{th} days) i.e. reduction of 11.48 % over 0.005 g/mL concentration. Lower concentration of *Juglans regia* L. leaf aqueous extracts (0.005 g/mL) were slightly stimulatory to root collar than control. In first 30 days, the root growth was slower than control, but later it grew faster and at 40^{th} day reached 7.34 mm (0.87 mm longer than control). At 40^{th} day, with 0.05 g/mL concentration the root collar diameter reached to 6.11 mm (94.43% of control). The fresh weight of aboveground, underground biomass and dry weight of *Isatis tinctoria* seedlings increased faster with *Juglans regia* L. leaf aqueous extract concentration of 0.005 g/mL than control. The *RI* values showed that the 0.05 g/mL extract concentration, slightly promoted the plants fresh weight, underground biomass and dry weight, but slightly inhibited the aboveground biomass. The magnitude of inhibitory effects increased with extract concentrations. After 40 days, the 0.1 g/mL extracts were inhibitory to aboveground biomass (14.06 % Inhibition) than control. The *RI* was -0.1233 and the strength of inhibition to fresh weight decreased to -0.1084.

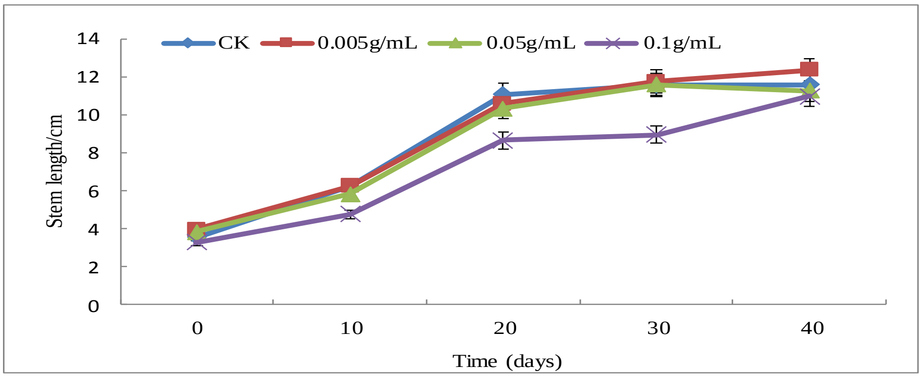

Figure 1. Effects of *J. regia* L. leaf aqueous extracts on stem length of *I. tinctoria*

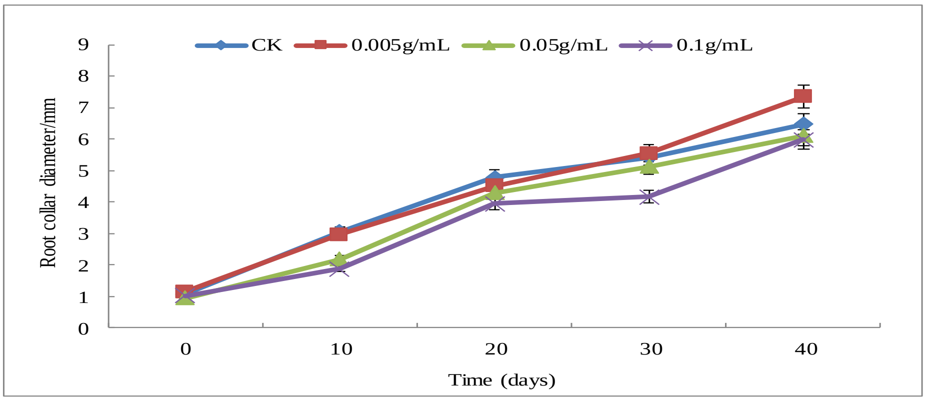

Figure 2. Effects of *J. regia* L. leaf aqueous extracts on root collar diameter of *I. tinctoria*

Table 1. Effects of *J. regia* L. leaf aqueous extracts on biomass of *I. tinctoria*

Aqueous Extract conc (g/mL)	Fresh weight/ plant		Shoot Biomass		Under ground biomass		Dry weight/ plant	
	(mg)	*RI*	(mg)	*RI*	(mg)	*RI*	(mg)	*RI*
Control	6.8374a		0.8809ab		0.4348b		1.3157bc	
0.005	8.9404a	0.2353	1.1094a	0.2060	0.5275a	0.1757	1.6306a	0.1931
0.05	7.3535a	0.0702	0.8757ab	-0.0061	0.5212a	0.1656	1.4032b	0.0624
0.1	6.0959b	-0.1084	0.7723b	-0.1233	0.3945b	-0.0927	1.1660c	-0.0032

RI: Response Index, Each value is mean of three replicates. Means within each column followed by the same letter are not significantly different at 5% level as per Duncan's multiple range test.

Photosynthesis

The different concentrations of aqueous extracts from *Juglans regia* L. leaf had variable effects on the photosynthesis in *Isatis tinctoria* seedlings (Table 2). At 0.005 g/mL concentration, the seedlings respiration and net photosynthesis rates were inhibited. The respiration rate was 2.5814 mol· $m^{-2}·s^{-1}$, which was reduced to 36.86 % than control. The net photosynthesis rate was 10.4722 µmol· $m^{-2}·s^{-1}$, which decreased to 35.88 % than control. The *RIs* were -0.3685 and -0.359 respectively. At 0.05 g/mL and 0.1 g/mL concentrations, the respiration rate and net photosynthesis rate were slightly inhibited. Respiration rates were 3.2160 mol· $m^{-2}·s^{-1}$, 3.6364 mol· $m^{-2}·s^{-1}$ and the *RIs* were -0.2133 and -0.1105 respectively. Net photosynthesis rates were 12.7945 µmol· $m^{-2}·s^{-1}$ and 11.6667 µmol· $m^{-2}·s^{-1}$. The *RIs* were -0.217 and -0.286 separately. Three concentrations (0.005 g/mL, 0.05 g/mL and 0.1 g/mL) of aqueous extracts inhibited the stomatal conductance and chlorophyll synthesis and promoted the intercellular CO_2 concentration of *Isatis tinctoria* seedlings. The *RI* of 0.005 g/mL concentrations was -0.276 and the stomatal conductance was reduced to 27.63 % than control (72.37 %). The stimulatory effects of intercellular CO_2 concentration increased with the increase in extract concentrations. At 0.1 g/mL extract concentrations, the intercellular CO_2 concentration reached 303.2500 µl/L i.e. increase of 10.03 % over control. At 0.005 g/mL, 0.05 g/mL and 0.1 g/mL extract concentrations, the chlorophyll concentration was 2.759, 2.568 and 2.402mg/g, respectively, and the *RIs* were -0.133, -0.193 and -0.245.

Table 2. Effects of *J. regia* L. leaf aqueous extracts on photosynthesis factors and chlorophyll contents of *I. tinctoria*

Aqueous Extract conc. (g/mL)	Respiration rate (m mol· $m^{-2}·s^{-1}$)		Net photosynthesis rate (µmol· $m^{-2}·s^{-1}$)		Stomatal conductance (mol $m^{-2}·s^{-1}$		Intercellular CO_2 conc (µl L^{-1})		chlorophyll content (mg/g)	
	Value	*RI*	Value	*RI*	Value	*RI*	Value	*RI*	Value	*RI*
CK	4.0883a		16.3306a		246.833a		272.8333a		3.183a	
0.005	2.5814b	-0.3685	10.4722b	-0.359	178.639a	-0.276	273.9167a	0.0040	2.759ab	-0.133
0.05	3.2160ab	-0.2133	12.7945ab	-0.217	201.195a	-0.185	280.2500a	0.0264	2.568b	-0.193
0.1	3.6364a	-0.1105	11.6667ab	-0.286	199.194a	-0.193	303.2500a	0.1004	2.402b	-0.245

RI: Response Index, Each value is mean of three replicates. Means within each column followed by the same letter are not significantly different at 5% level as per Duncan's multiple range test.

Aqueous extracts of some plants decreased the chlorophyll content, slowed the photosynthetic rate and inhibited the seedling growth and development (7,23,36). The aqueous extracts from *Ailanthus* root inhibited the growth of *Robinia* seedling, due to decrease in chlorophyll content and biomass (3). Jia *et al*. (16) found that the mechanism of allelopathic effects in *Pinus tabuliformis-Quercus liaotungensis* mixed forest was through the soil leachates, which influenced the photosynthesis. In our experiment, the chlorophyll content of *Isatis tinctoria* seedlings decreased with increasing concentration.

Physiological parameters

The *Juglans regia* L. leaf aqueous extracts decreased the soluble protein contents of *Isatis tinctoria* (Fig. 3). The soluble protein content decreased with treatment time. After 30 days, the inhibition rates of 0.005 g/mL, 0.05 g/mL and 0.1 g/mL concentrations decreased the soluble protein contents by 11.30 %, 12.13 % and 12.78 %, respectively, than control. The *Juglans regia* L. leaf aqueous extracts concentrations and treatment time significantly influenced the soluble sugar contents of *Isatis tinctoria* (Fig. 4). The soluble sugar content

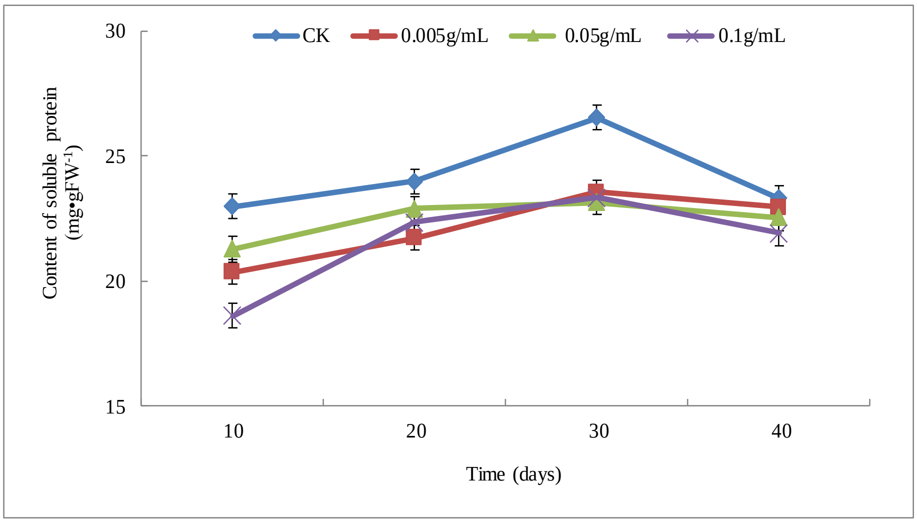

Figure 3. Effects of *J. regia* L. leaf aqueous extracts on the soluble protein content of *I. tinctoria*

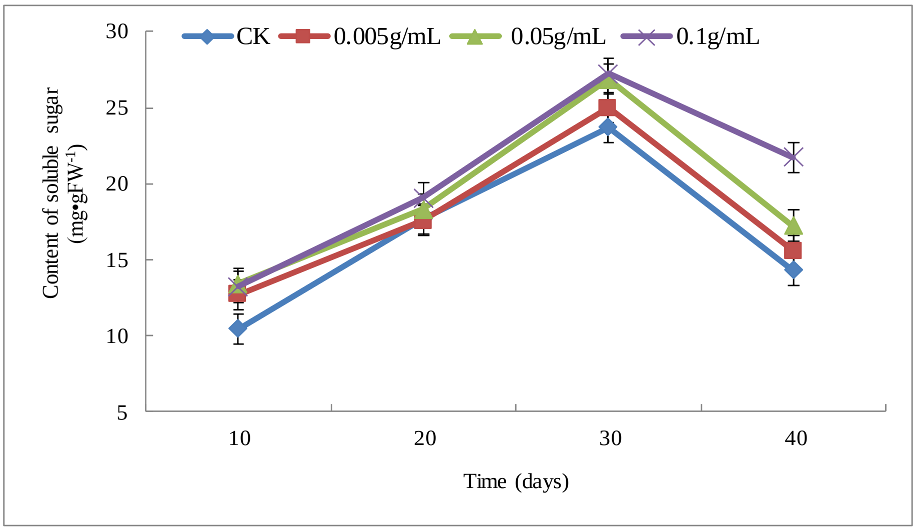

Figure 4. Effects of *J. regia* L. leaf aqueous extracts on soluble sugar content of *I. tinctoria*

of *I. tinctoria* increased with extract concentrations from 10 to 30 days and decreased thereafter. The sugar content at 30th day with 0.005 g/mL, 0.05 g/mL and 0.1 g/mL concentrations reached 24.97 mg·gFW^{-1},26.87 mg·gFW^{-1} and 27.25 mg·gFW^{-1} respectively. The different concentrations of aqueous extracts of *Juglans regia* L. significantly influenced the PRO and MDA contents of *Isatis tinctoria* seedlings (Fig. 5, Fig. 6). After 30th days, the PRO content reached the highest and the contents in control, 0.005 g/mL, 0.05 g/mL, 0.1 g/mL were 0.236 %,0.388 %, 0.453 % and 0.477% respectively. The MDA content increased with the increase in aqueous extracts concentration and it followed the pattern similar to PRO content i.e. increased first and then decreased. After 30 days, MDA content reached the highest and the contents in control, 0.005 g/mL, 0.05 g/mL, 0.1 g/mL and increased by 3.17 %, 50.18 % and 57.93 % respectively. These results were similar to former report showing that *Citrullus lanatus* extracts promotes the MDA content in *Lactuca sativa* leaves (10).

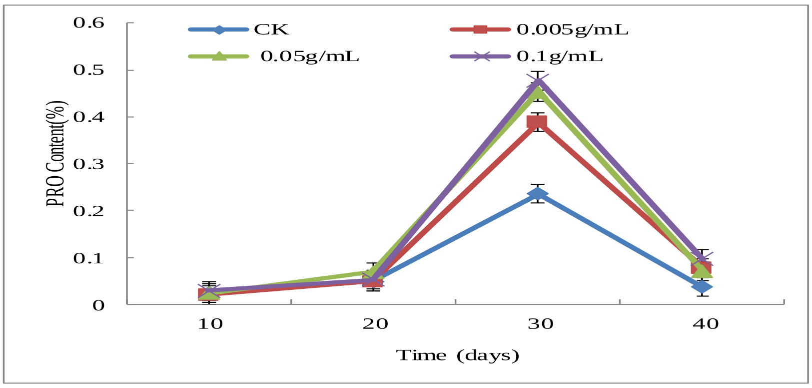

Figure 5. Effects of *J. regia* L. leaf aqueous extracts on PRO of *I. tinctoria*

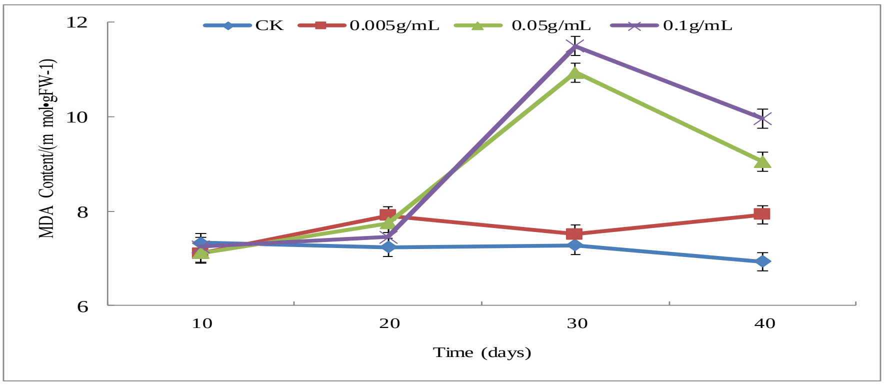

Figure 6. Effects of *J. regia* L. leaf aqueous extracts on the MDA content of *I. tinctoria*

The different concentrations of aqueous extracts of *Juglans regia* L. leaf variably influenced the POD, SOD and CAT contents of *Isatis tinctoria* (Fig. 7, Fig. 8, Fig. 9).

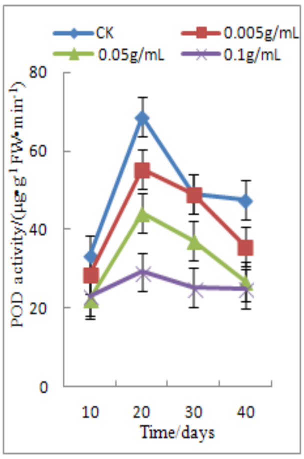

Figure 7. Effects of *J. regia* leaf aqueous extracts of POD activity of *I. tinctoria*

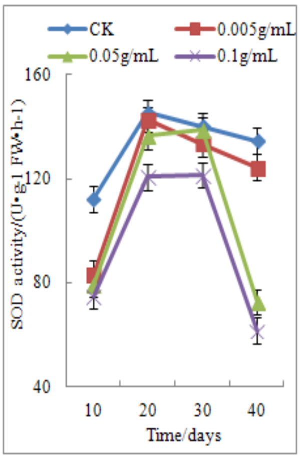

Figure 8. Effects of *J. regia* leaf aqueous extracts on SOD activity of *I. tinctoria*

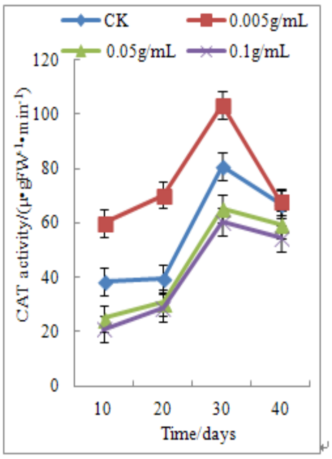

Figure 9. Effects of *J. regia* leaf aqueous extracts on the CAT activity of *I. tinctoria*

(i). POD: At the same concentration, the POD activity increased from the 10 to 20 days, and then decreased. After 20 days, the POD activities at 0.005 g/mL, 0.05 g/mL and 0.1 g/mL *Juglans regia* L. leaf aqueous extract reached lowest and the inhibition rates were 19.36 %, 35.72 % and 57.46 %, respectively.

(ii). SOD: The application of *Juglans regia* L. leaf aqueous extracts had variable effects on the SOD activity of *Isatis tinctoria* seedlings. The SOD activity decreased with the increasing concentrations. After 40 days, at 0.005 g/mL, 0.05 g/mL and 0.1 g/mL extract concentrations, the SOD activity inhibition rates were 7.55%, 46.02% and 54.19% than control, respectively. The SOD activity increased first and then decreased. At 0.005 g/mL extraction concentration, the SOD activities reached the highest on 20th day and remained constant till 30 days and decreased thereafter. The value of SOD activities were 145.38 U·g^{-1}FWh^{-1} at 20th day and 142.73 U·g^{-1}FWh^{-1} at 30th day with the 0.005 g/mL extraction concentration. At the end of growth period (40th day), the SOD activities in all extract concentrations decreased to normal level.

(iii). CAT: The application of *Juglans regia* L. leaf aqueous extracts at all concentrations increased the CAT activity till 30 days and decreased afterwards. The CAT activity reached the highest (27.84 % increase over control) on 30th day. The higher concentrations (0.05 g/mL and 0.1 g/mL) of extract treatments, inhibited the CAT activities. At 40th day, the 0.1 and 0.5 g/mL extract concentrations the inhibition rates decreased to 11.21 % and 18.90 %, respectively.

Allelopathy Mechanism of *Juglans regia* L. leaf extracts on *Isatis tinctoria*

Many researches had shown that the aqueous extracts affects the physiological and biochemical processes in plants (26,33,39). In terms of the synthetic effect, *Juglans regia* L. extract inhibited the POD, SOD and CAT activities of *Isatis tinctoria* seedlings, which suggested that the *Juglans regia* L. leaf extracts might break the normal defence system, decrease the ability to remove H_2O_2, block the physiological and biochemical processes and thereby inhibit the seedling growth.

Table 3. *I. tinctoria* Synthetic analysis of allelopathic effects mechanism of *J. regia* L. leaf aqueous extracts

Function mechanism	Inhibition (%)			Synthetic effect	Allelopathic effects
	0.005 (g/mL)	0.05 (g/mL)	0.1 (g/mL)		
Soluble protein	8.51	7.19	10.99	8.90	Content decreased
Soluble sugar	-7.07	-14.69	-22.87	-14.88	Content increased
PRO	-54.51	-78.25	-89.88	-74.21	Content increased
MDA	-5.79	-21.09	-25.67	-17.52	Content increased
POD	15.23	34.56	48.46	32.75	Activity decreased
SOD	9.07	19.73	28.86	19.22	Activity decreased
CAT	-33.49	20.04	27.09	4.55	Activity decreased

The aqueous extracts from rice leaves drastically inhibit the SOD and POD activities of *Echinochloa crusgalli*, but increased the MDA content (13). Lin et al. (19) showed that autotoxins greatly influenced the cucumber root SOD and POD activities and increased the oxygen content in root tissues. The allelochemicals in aqueous extracts of *Juglans regia* L. leaf might suppress the soluble protein content, promote the soluble sugar, PRO and MDA, break the protective enzyme system and the cell membrane and increase the cell membrane permeability. The mechanism of aqueous extracts not only depended on the concentrations, but also was related to their combination and environment (18). In this study, we found that *Juglans regia* L. leaf extracts have double effects on seedling growth. The lower concentrations of *Juglans regia* L. leaf extracts had stimulatory effects and the higher concentration were inhibitory to fresh weight, shoot biomass, underground biomass and dry weight of *Isatis tinctoria.*. We suggest that the changes in chlorophyll and photosynthetic factors of *Isatis tinctoria* were one of the physiological mechanisms of the allelopathic effects of *Juglans regia* L. leaf extracts. There were some water-soluble allelochemicals in *Juglans regia* L. leaf, However, the type and mass concentration of allelochemicals in *Juglans regia* L. leaf are still unclear, which requires further research.

ACKNOWLEDGEMENTS

This research is supported by the Major Innovation Projects for Building First-class Universities in China's Western Region (No.: ZKZD2017004) and National Natural Science Foundation of China (No.: 31660133). Many thanks are given to Mr. Zh. J. Sun, Mr. Sh. X. Zhang, Dr. F. Chen and Dr. L. Wang, for their help in field experiments.

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