

## Effects of walnut (*Juglans regia* L.) root exudates on germination, seedling growth and enzymatic activities of turnip (*Brassica rapa* L.)

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### ABSTRACT

To determine the allelopathic effects of root exudates of walnut trees, the root exudates of 2, 3, and 4-years-old walnut (*Juglans regia* L.) trees were extracted with the neutral, acidic (pH 3.0), and alkaline (pH 8.0) components of ethyl acetate extract. The bioactivity of these 3-components (neutral, acidic and alkaline) were separately investigated on the seed germination, seedling growth, the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and the content of malondialdehyde (MDA) of turnip (*Brassica rapa* L.). The 3-components of ethyl acetate extract of root exudates of walnut trees inhibited the seed germination and seedling growth of turnip as compared to control. Of all the enzymes assayed (SOD, POD, CAT and MDA), the SOD and the POD activity played a cooperative effect in clearing the reactive oxygen radicals. In 3-treatments (neutral, acidic and alkaline) of root exudates of 4-years-old walnut trees, the activities of SOD, POD and CAT enzymes in turnip were higher than the 3-treatments of root exudates of 2 and 3-years-old walnut trees, whereas the activity of MDA was lower. Thus *in-vitro* allelopathic effects of walnut root exudates were more pronounced with the increasing age of walnut trees. The *in-vitro* inhibitory/stimulatory effects of root exudates of walnut trees varied with the pH of ethyl acetate extract and with the age of walnut trees.

**Key words:** Allelopathy, bioassay, *Brassica rapa* L., catalase, *Juglans regia* L., malondialdehyde, peroxidase, physiology, root exudates, superoxide dismutase, walnut.

### INTRODUCTION

Allelopathy has been defined as all effects of plants on neighboring plants through the release of chemical compounds into the environment (17). Allelopathy is part of interference mechanism in which living or dead plants release allelochemicals exerting an effect (mostly negative) on associated plants. Allelopathy can play an important role in natural and managed ecosystems (7,9). Allelochemicals are low molecular weight compounds excreted from plants during the secondary metabolism (18). The germination and growth of plants from the undergrowth can be inhibited by these compounds which are present in other plant species or in their residues, either directly or by microbiological activity in the soil. These substances are released into the environment through root exudates, leaching of aerial parts, decomposition of vegetable residues and volatilization of

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compounds. These compounds vary in chemical composition, concentration and localization in plant tissues and from plant to plant with changes in both biotic and abiotic conditions (9). Allelochemicals produced by one crop can affect the growth, productivity and yield of other crops or same crop (1).

The black walnut (*Juglans nigra* L.) is most notorious allelopathic tree. Its plants produce a non-toxic colourless chemical compound called hydrojuglone (8,14,16). Black walnut allelopathy adversely affects the growth of vegetables, field crops, ornamental plants and several woody species (19,20,21). Walnut leaves are source of healthcare compounds and are used in traditional medicine to treat venous insufficiency and haemorrhoidal symptomatology, and for its antidiarrheic, antihelminthic, depurative and astringent properties (3,27,29). Its keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scorfulous and sedative activities are also known (26). *J. regia* L. is less toxic than *J. nigra*. The potatoes, beans and alfalfa do not grow well beneath the walnut. In northern India, *J. regia* is commonly grown along bunds and terraces of cultivated fields and there is low species richness underneath the canopy than in open areas (30). In China and in northern India, agroforestry systems are adopted and walnut is most economical tree specie in this practice. Its characteristics of high value, aesthetic qualities, production capacity, rapid growth potential and adaptability to management makes the specie very suitable for intercropping (24).

Most assessments of allelopathy or autotoxicity involve bioassays of plant or soil extracts based on seed germination and seedling growth. However, the mechanism and allelochemicals involved in root exudates of walnut remain scarce. To better understand the mechanism of walnut autotoxicity or allelopathy we evaluated the effects of walnut root exudates on seed germination, seedling growth, protective enzyme systems and the malondialdehyde content of turnip (*Brassica rapa* L.). This study aimed to test *in-vitro* allelopathic effects of root exudates of walnut trees on turnip metabolism, get detailed information about the allelopathic relationships between walnut and test plants to elucidate the physiological effects of allelochemicals and provide more evidence of walnut allelopathy.

## MATERIALS AND METHODS

### Walnut root exudates

Thirty actively growing walnut (*Juglans regia* L.) trees (2, 3 and 4 years old) were selected from the Nursery Garden of our University as per method of Liao *et al.* (12). Then the lignified branch roots of selected trees were dug out and exposed and the damaged roots were not used. Selected roots were not separated from the actively growing walnut trees, but all the fibrous roots of selected branch roots were removed. Then the branch roots were enclosed into nylon mesh bags (filled with soil) and were buried in soil. One month later, the branch roots were dug up (taking care not to damage new rootlets). They were rinsed clean, first with tap water and then with distilled water. Thereafter the new roots were set into sterile conical flasks and these were sealed. Five months later, the conical flasks were opened and their root exudates were collected. During the whole process, the conical flasks were kept free of contamination, to reduce microbial infection (12).

We obtained 200 mL root exudate of 2, 3 and 4-years-old walnut (*J. regia* L.) trees. Then the root exudates of walnut trees were filtered using suction flask and buchner funnel to remove particulate debris. Forty-five ml ethyl acetate was used as the extracting agent to obtain neutral component. The pH value of aqueous phase was then adjusted to 3 with 1 mol·L<sup>-1</sup> HCl, then 45 mL ethyl acetate was added again to obtain acidic component. The pH value of aqueous phase was then adjusted to 8 with 1 mol·L<sup>-1</sup> NaOH, ethyl acetate was added to get alkaline component. The acidic, alkaline and neutral components of the ethyl acetate extracted root exudates of walnut trees were concentrated to the final volume of 5 mL (35 °C) (34). Two ml was used to evaluate the effects on seed germination, seedling growth, protective enzyme systems and on the malondialdehyde level of test plants.

#### Bioassay

Seeds of turnip (*Brassica rapa* L. ssp. *rapifera* (Metzg.) Sinsk. were obtained from College of Horticulture of our University, these were surface-sterilized by immersing for 10 min in 95% (v/v) ethanol followed by 30 min in 0.3 % hydrogen peroxide. Seeds with cracks in seed coat and internal microbial contamination absorb water more quickly than intact seeds and these float on the water surface, where they were collected and discarded. The remaining seeds were rinsed 5 times with sterile, double-distilled water (ddH<sub>2</sub>O). Two ml of concentrated acidic, alkaline and neutral ethyl acetate extract, were separately added to two-layers of filter paper in sterile Petri dish (11 cm dia) and distilled water was used as control. When the ethyl acetate extract had volatilized, 30 sterilized seeds were evenly spaced on the filter paper in the sterile Petri dishes. Then all the Petriplates were kept in an incubator in dark at 25 ± 1 °C. Treatments were arranged in a completely randomized design with three replications. Germination was determined by counting the number of germinated seeds at 24-h intervals till 10 days. After 10 d incubation, the number of germinated seeds in each dish was recorded, the length of root and shoot of seedlings were measured. Then the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activity and malondialdehyde (MDA) of receptor plants were measured.

**SOD activity:** Superoxide dismutase (SOD) activity was measured using the nitroblue tetrazolium (NBT) reduction method of Zou (35). About 0.5 g turnip leaves, treated with walnut root exudates were grounded into powder in the precooled mortar, followed by 2 mL phosphate buffer (50 mmol·L<sup>-1</sup>, at pH 7.8). When the sample was sufficiently homogenized, four ml phosphate buffer was added to wash the mortar, then, additional phosphate buffer to 10 mL. The solution was transferred into a plastic centrifuge tube, centrifuged at 10000 r·min<sup>-1</sup> for 20 min at 4 °C by refrigerated centrifuge (SIGMA). The SOD reaction system consisted of following components: 1.5 mL phosphate buffer (50 mmol·L<sup>-1</sup>, at pH 7.8), 0.3 mL methionine (Met) (130 mmol·L<sup>-1</sup>), 0.3 NBT (750 µmol·L<sup>-1</sup>), 0.3 mL EDTA-Na<sub>2</sub> (100 µmol·L<sup>-1</sup>), 0.3 mL riboflavin (20 µmol·L<sup>-1</sup>), 0.1 mL enzyme solution and 0.5 mL distilled water. The SOD activity was determined by U-2001 spectrophotometer (HITACHI) by using colorimetric determination at 560 nm. The activity of SOD is given as U·g<sup>-1</sup>FW·min<sup>-1</sup>. After centrifugation, the supernatant was used in separate assays of POD activity and CAT activity.

**POD activity:** The peroxidase (POD) activity was assayed as per method of Zou (35). The POD reaction system consisted of following components: 0.9 mL guaiacol ( $20 \text{ mmol}\cdot\text{L}^{-1}$ ), 1 mL phosphate buffer ( $50 \text{ mmol}\cdot\text{L}^{-1}$ , pH 7), 0.1 mL enzyme solution and 1 mL  $\text{H}_2\text{O}_2$  ( $30 \text{ mmol}\cdot\text{L}^{-1}$ ). The POD activity was determined by U-2001 spectrophotometer (HITACHI) at 470 nm. The activity of POD was calculated by recording the change in  $\text{OD}_{470}$  values at 3 min intervals after the initial biochemical reaction. POD activity was shown as  $\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{min}^{-1}$ .

**CAT activity:** The catalase (CAT) activity was determined as per method of Zou (35). The reaction system for CAT included the following components: 1.9 mL phosphate buffer (pH 7,  $50 \text{ mmol}\cdot\text{L}^{-1}$ ), 0.1 mL reserved supernatant (the same as for SOD activity assay) and 1 mL distilled water. The colorimetric determination of CAT was conducted by U-2001 spectrophotometer (HITACHI) at 240 nm. The biochemical reaction was started by adding 0.3 mL  $\text{H}_2\text{O}_2$  ( $30 \text{ mmol}\cdot\text{L}^{-1}$ ) in the reaction system. The CAT activity was calculated by changes in  $\text{OD}_{240}$  values at 3 min intervals after the initial biochemical reaction and the units shown are  $\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{min}^{-1}$ .

**MDA activity:** Level of lipid peroxidation was expressed as the content of MDA as per Zhang *et al.* (33). About 0.3 g leaves were grounded into powder in the precooled mortar, followed by 2 mL phosphate buffer ( $50 \text{ mmol}\cdot\text{L}^{-1}$ , at pH 7.8). When the sample was sufficiently homogenized, 3 mL phosphate buffer was added to wash the mortar, added 5 mL Thiobarbituric acid (TBA) ( $50 \text{ mmol}\cdot\text{L}^{-1}$ ) and thereafter, all solution was boiled in water bath for 10 min, then transferred into a plastic centrifuge tube, centrifuged at  $3000 \text{ r}\cdot\text{min}^{-1}$  for 20 min. The supernatant after centrifugation was used to measure the MDA activity assay. The MDA activity was determined by U-2001 spectrophotometer (HITACHI) by using Colorimetric Determination with U-2001 spectrophotometer (HITACHI) at 600 nm, 532 nm and 450 nm colorimetric wavelength. The activity unit of MDA is given as  $\text{mmol}\cdot\text{g FW}^{-1}$ .

#### Data analysis

The statistical analysis of all data was done using one-way analysis of variance (ANOVA) test in SPSS 16.0. Differences among means were detected with Duncan's multiple range test at 5% level.

## RESULTS AND DISCUSSION

#### Seed germination

The three components (neutral, acidic, and alkaline) of the ethyl acetate extract of the root exudates of walnut trees (2, 3, and 4 years old) were individually distinctive in the growth responses of test plant. The germination rate of turnip seeds decreased in the three treatments compared to the control (Table 1). Neutral treatment showed the highest inhibition (51.6%) in turnip seeds germination rate by root exudates of four-years-old the walnut trees although it was not statistically proven whereas alkaline treatment showed a significant reduction in germination rate ( $P < 0.05$ ). Contrary, in acidic treatment the germination rate was mostly inhibited (54.7%) when treated by root exudates of

two-year-old walnut trees (Fig. 1). The germination rate of turnip seeds, treated with the neutral component of the ethyl acetate extract of the root exudates of four-year-old walnut trees, was lower than other two but significantly lower than treated with the acidic component and alkaline component ( $P < 0.05$ ). In general, a four-year-old walnut tree showed the greatest inhibition in all treatments (neutral, acidic, and alkaline), with only one exception- acidic component of root exudates of two-year-old walnut tree. And the mean germination rate of turnip seeds in acidic component was significantly ( $P < 0.05$ ) lower than other two components.

Table 1. Effects of different pH of root exudates of Walnut trees of 2,3,4 years age on the germination rate of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	Germination rate (%)		
	Neutral	Acidic	Alkaline
Control	0.95±0.11a	0.95±0.11a	0.95±0.11a
2	0.75±0.01a	0.43±0.02ab	0.61±0.06b
3	0.73±0.01a	0.77±0.04ab	0.71±0.02b
4	0.46±0.10a	0.49±0.01b	0.57±0.03ab
Mean	0.72±0.03a	0.66±0.10ab	0.71±0.09b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup> Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

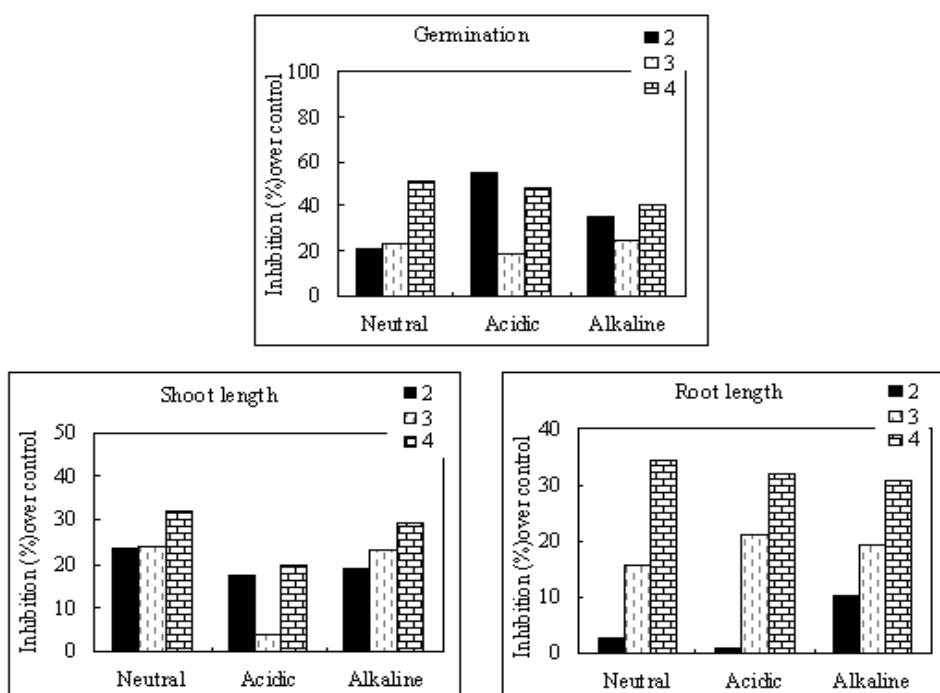


Figure 1. Effects of root exudates of different pH of walnut trees of 2,3,4 years old on seed germination, shoot length and root length of turnip.

Our results imply that allelopathic activity depend on the different components (pH value) of the ethyl acetate extract of the root exudates of walnut trees, and that the root exudates of four-year-old walnut trees showed higher inhibitory effect. This might be ascribed to different allelopathic effects resulting from component variability of allelochemicals during the aging of walnut trees.

### Seedling growth

The seedling growth of turnip decreased in the three treatments compared to the control (Table 2). Neutral, acidic and alkaline components of the ethyl acetate extract of root exudates showed the highest inhibition of shoot length by four-years-old walnut tree (Table 2, Fig. 1). The shoot length of turnips treated with the neutral component of the ethyl acetate extract of the root exudates of walnut trees (2, 3, and 4 years old) was significantly inhibited as compared with treated with the acidic component respectively ( $P < 0.05$ ). And the four-years-old walnut tree showed the greatest inhibition in all treatments (neutral 32.2%, acidic 19.8%, and alkaline 29.4% respectively). These results suggest that of all the three components of the ethyl acetate extract of the root exudates of walnut trees, the neutral component may play more important role in the allelopathy effect than the other components, and as the age of the walnut trees was longer, the allelopathy effect of ethyl acetate extract of their root exudates was more pronounced.

Table 2. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the shoot length of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	Shoot length (cm)		
	Neutral	Acidic	Alkaline
Control	7.69±0.01ab	7.69±0.01ab	7.69±0.01ab
2	5.89±0.09a	6.37±0.10ab	6.22±0.21a
3	5.85±0.07a	7.39±0.03a	5.92±0.19ab
4	5.21±0.02a	6.17±0.06b	5.43±0.05ab
Mean	6.16±0.05ab	6.91±0.10b	6.32±0.12b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup> Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

Earlier studies reported that extracts of allelopathic plants had more pronounced effects on radical growth than on hypocotyls growth or shoot growth (4). Such an outcome might be expected, because it is likely that roots are the first to absorb the allelochemicals or autotoxic compounds from the environment (25). In this experiment, we found that the root length of turnips was also sensitive under the three treatments of walnut root exudates compared with the shoot length. Inhibitory effect of the root length of turnips, treated by the components of the ethyl acetate extract of the root exudates of four-year-old walnut trees, was found as compared with that of two- and three-year-old walnut trees in relation to control (Table 3). The inhibition was similar to the root length of turnips, treated by the three components of the ethyl acetate extract of the root exudates of four-year-old walnut trees, as compared to that of two-year-old walnut trees (Figure 1). The highest inhibition of the root length of turnips were respectively found under the neutral treatment, acidic treatment and alkaline treatment of the root exudates of four-year-old walnut trees (34.5%, 32.0%, and 30.6% respectively). Alkaline component showed the lowest level in the mean

root length of turnip, which indicated that alkaline component play more important role in the allelopathy effect. These may lead to the same conclusion on the effect to the shoot length of turnips that as the age of the walnut trees was longer, the allelopathic effect of ethyl acetate extract with root exudates of walnut trees was more significant.

Table 3. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the root length of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	Root length (cm)		
	Neutral	Acidic	Alkaline
Control	9.37±0.08a	9.37±0.08a	9.37±0.08a
2	9.14±0.15a	9.29±0.14ab	8.40±0.18ab
3	7.88±0.11b	7.41±0.12a	7.55±0.07ab
4	6.14±0.10a	6.37±0.05ab	6.50±0.21b
Mean	8.13±0.11a	8.11±0.09ab	7.96±0.14b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup> Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

Allelopathy has been claimed to exist between numerous species (6,10,11,15). The chemical responsible for walnut allelopathy is juglone (5-hydroxy-1, 4 naphthoquinone) (5). Juglone allelopathic effects on plants are generally toxic but beneficial to some species. Two major flavonol allelochemicals [kaempferol (H) and kaempferol-3-O- $\alpha$ -L-arabinofuranoside (I)] were isolated in walnut leaves extracts (32). This is one of the first observations of allelopathic properties in the walnut root exudates. Such effects have been reported in fir trees [*Cunninghamia lanceolata* (Lamb.) Hook] (12). In our experiment, the root exudates of walnut trees (2, 3, and 4 years old) inhibited seed germination and seedling growth of turnips as compared with control and the acidic, alkaline, and neutral components of the ethyl acetate extract of the root exudates of walnut trees exhibited variable allelopathic activities on seed germination and seedling growth of turnips.

### Enzyme activities

**SOD activity:** The activity of SOD in turnips, whose seeds were sown in three components (neutral, acidic, and alkaline) of the ethyl acetate extract of root exudates of 3-years-old walnut trees for 10 d, was respectively lower than 2 and 4-years-old walnut trees (Table 4), which indicated the strongest inhibition: 18.2%, 13.8%, and 19.1%, respectively (Fig. 2). SOD activity of turnips, treated with the alkaline components of the ethyl acetate extract of the root exudates of 4-years-old walnut trees, was the highest and significantly differed in relation to control together with acidic component of 2-years-old walnut tree ( $P < 0.05$ ), which showed the lowest inhibition: -0.8% and 0.2%, respectively. Activity of SOD in turnips, treated with the neutral and alkaline components of the ethyl acetate extract of the root exudates of 4-years-old walnut trees, was higher than that of 2- and 3-years-old walnut trees respectively, whereas the activity of SOD in turnips, treated with the acidic components of the ethyl acetate extract of the root exudates of 2-years-old walnut trees, was higher than that of 3- and 4-years-old walnut trees. Statistically significant differences were found between neutral and acidic components of the ethyl acetate extract of root exudates of 3-years-old walnut trees. While the activity of SOD in turnips, treated with the alkaline

component of the ethyl acetate of the root extracts of 4-years-old walnut trees was higher than treated by other two components, statistical difference was found between alkaline and acidic components. The activity of SOD in turnips, treated with neutral component of the ethyl acetate of the root extracts of 4-year-old walnut trees was lower than treated by other two components. And the mean content of SOD activity of turnip in alkaline treatment of root exudates of walnut trees, was lower than other two treatments (neutral and acidic), which showed the significant allelopathy effect. Different root exudates treatments from walnut trees in different ages varied in their ability to influence SOD activity.

Table 4. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the SOD Activity of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	SOD activity ( $U \cdot g^{-1}FW \cdot min^{-1}$ )		
	Neutral	Acidic	Alkaline
Control	25.92±0.08a	25.92±0.08a	25.92±0.08a
2	24.68±0.48a	25.87±0.67a	23.34±0.14ab
3	21.21±0.79a	22.35±0.15b	20.96±0.76a
4	24.73±0.33ab	25.23±0.03a	26.15±0.85b
Mean	24.14±0.42a	24.84±0.23a	24.09±0.46b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup> Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

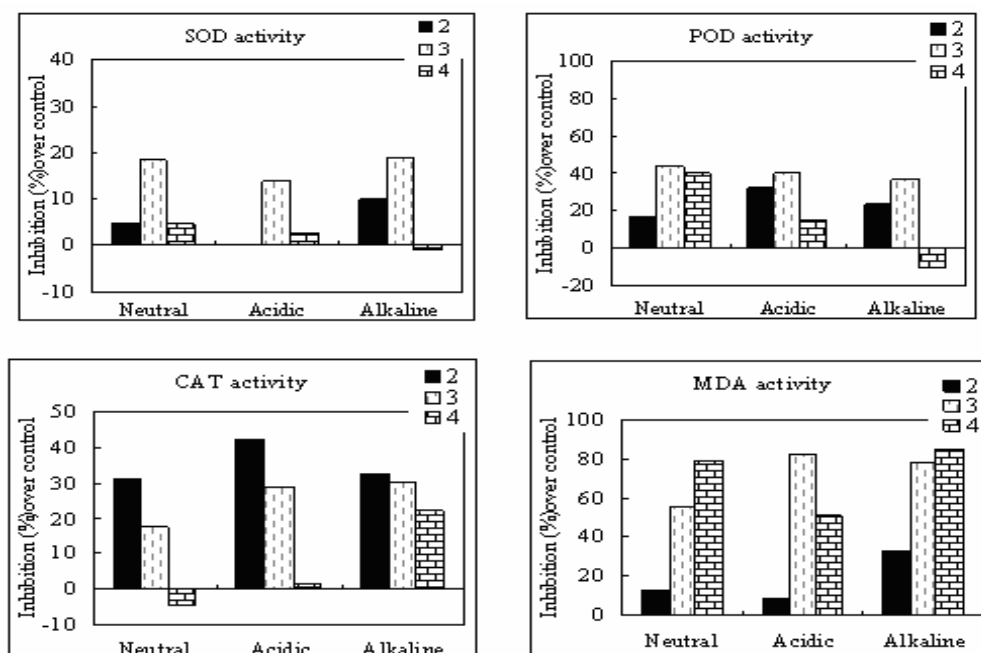


Figure 2. Effects of root exudates of different pH of walnut trees of 2,3,4 years old on activities of enzymes of turnip.

**POD activity:** The finding in our study indicated that the POD activity of turnips, treated with the three components (neutral, acidic, and alkaline) of the ethyl acetate extract of root exudates of 3-years-old walnut trees, was respectively lower than 2- and 4-years-old walnut trees (Table 5), which showed the strongest inhibition: 43.5%, 39.7%, and 36.9% respectively (Figure 2). Statistical significance ( $P < 0.05$ ), was found in the activity of POD in turnips, treated with the acidic component between 2-years-old and 3-years-old walnut trees. Significant differences in POD activity were found between neutral and other two treatments (acidic and alkaline) of the ethyl acetate extract of the root exudates of 2-years-old walnut trees. Highest activity of POD in turnips, treated with the neutral components of the ethyl acetate extract of the root exudates of 2-years-old walnut trees, in relation to 3- and 4-years-old walnut trees was found ( $P < 0.05$ ). Contrary, the activity of POD in turnips, treated with the acidic and alkaline components of the ethyl acetate extract of root exudates of 4-years-old walnut trees, was higher in relation to 2- and 3-years-old walnut trees, which indicated the lowest inhibition: 15.1%, -10.0% as compared to the control respectively. The POD activity in neutral component was lowest in 3- treatments of root exudates of walnut trees, which showed neutral component may contain some compound causing the allelopathic effect.

Table 5. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the POD Activity of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	POD activity ( $\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{min}^{-1}$ )		
	Neutral	Acidic	Alkaline
Control	1269.41±0.22ab	1269.41±0.22ab	1269.41±0.22ab
2	1055.17±0.23a	855.48±0.74b	975.50±0.53b
3	716.67±1.05b	765.03±0.96a	800.37±0.43b
4	761.94±0.61b	1078.33±0.03ab	1396.69±0.11b
Mean	950.79±0.53ab	992.06±0.49ab	1110.5±0.32b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup> Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

**CAT activity:** The CAT activity of turnip decreased in all three treatments compared to the control, except in treatment with neutral component of root exudates of 4-years-old walnut tree ( $P < 0.05$ ), Table 6, which showed the lowest inhibition: -4.3% (Figure 2). Therefore, the CAT activity of turnips treated with the neutral component of the ethyl acetate extract of the root exudates of 4-years-old walnut trees was higher than treated with the other two components (acidic and alkaline). In general, CAT activity showed increasing tendency with aging of walnut trees in all treatments. The CAT activity of turnips, treated with the acidic component of ethyl acetate extract of root exudates of 2-years-old walnut trees, showed the strongest inhibition: 42.4%, but was the lowest than other two components (neutral and alkaline), (Table 6). The alkaline component in mean content of CAT activity showed the lowest level in the three treatments of the root exudates of walnut trees, which showed the obvious allelopathy effect, similar to that of SOD activity.

Table 6. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the CAT Activity of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	CAT activity ( $\text{U}\cdot\text{g}^{-1}\text{FW}\cdot\text{min}^{-1}$ )		
	Neutral	Acidic	Alkaline
Control	97.24±0.39a	97.24±0.39a	97.24±0.39a
2	67.01±0.35a	55.98±0.23ab	65.36±0.78a
3	80.28±0.96a	69.28±0.53a	67.64±0.69b
4	101.42±0.42b	95.95±0.95a	75.88±0.87a
Mean	86.49±0.53a	79.61±0.50a	76.53±0.68ab

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup>Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

**MDA activity:** The MDA activity of turnips, treated with all the three treatments (neutral, acidic, and alkaline) of the root exudates of walnut trees (2, 3, and 4 years old), was lower than control (Table 7). Neutral and alkaline treatments showed significant reduction ( $P < 0.05$ ) in MDA activity of turnip with aging of walnut trees, which showed the strongest inhibition (78.8% and 84.9%, respectively) in 4-years-old walnut trees (Figure 2). Contrary, acidic treatment showed the highest inhibition (82.6%) of MDA activity by root extracts of 3-years-old walnut trees than 2- and 4-years-old respectively ( $P < 0.05$ ). Unlikely to SOD, POD and CAT, the MDA activity of turnips, treated with the three components of the ethyl acetate extract of the root exudates of 2-years-old walnut trees, was significantly higher than 3- and 4-years-old walnut trees, respectively. In alkaline component, the MDA activity was at lowest level in three treatments of root exudates of walnut trees, which was similar to SOD activity and CAT activity.

Table 7. Effects of the root exudates (RE) of walnut trees of 2,3,4 years age on the MDA Activity of turnip at 10 DAS ( $n = 9$ )

Root exudates of Walnut trees age (Years)	MDA activity ( $\text{mmol}\cdot\text{g}\text{FW}^{-1}$ )		
	Neutral	Acidic	Alkaline
Control	10.7±0.59b	10.7±0.59b	10.7±0.59b
2	9.35±0.35a	9.81±0.57a	7.25±0.22a
3	4.84±0.44b	1.86±0.15ab	2.34±0.03ab
4	2.27±0.27b	5.24±0.24b	1.62±0.12b
Mean	6.79±0.41ab	6.90±0.39b	5.48±0.24b

Each value is mean of three replicates. DAS: Days after sowing

<sup>a, b</sup>Different letters in rows indicate significant differences (significance level of  $P < 0.05$ )

To resist oxidative stress, plants can induce a series of detoxification reactions catalyzed by antioxidative enzymes, including CAT, SOD and POD (28,33). The activity of enzymes in the protective system changes when stimulated by exogenous toxic material. The protective enzyme systems (SOD, POD, and CAT), are the main enzymes to scavenge the reactive oxygen species, can inhibit the peroxidation of cellular membrane lipids. SOD can turn  $\text{O}_2^{-1}$  to  $\text{H}_2\text{O}_2$  through dismutation, then CAT and POD turn  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$ . This series of reactions can eliminate biomembrane damaged by  $\text{O}_2^{-1}$ . MDA is one of the ultimate products as a result of lipid peroxidation damage by free radicals. MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from the oxydative stress.

Generally, the activities of SOD, CAT, and POD increase with time, when reaching the highest value at some time, they decrease sharply. Our experiments showed general increase in the activity of protective enzyme SOD as well as the POD in turnips, treated with the three components (neutral, acidic, and alkaline) of the ethyl acetate extract of the root exudates of 4-years-old walnut trees, with exceptions of all treatments with extracts of 3-years-old walnut for SOD and POD, and the alkaline component of root exudates of 4-years-old walnut for SOD and POD, was respectively higher, than 2- and 3-years-old walnut trees, indicating that the two enzymes played a cooperative role in clearing the reactive oxygen radicals. When turnips were treated with the three components (neutral, acidic, and alkaline) of ethyl acetate extract of root exudates of 4-years-old walnut trees, the activity of CAT in the turnips was respectively higher than 2 and 3-years-old walnut trees. However, the activity of MDA in the turnips treated with the neutral and alkaline components of ethyl acetate extract of the root exudates of 4-years-old walnut trees was lower than treated with 2- and 3-years-old walnut trees, with exception of acidic component of root exudates of 4-years-old walnut tree. The present results implies that the age of walnut trees and the different components of the ethyl acetate extract of root exudates of walnut trees can serve as an elicitor to induce higher allelopathic potential of donor walnut to suppress the target plants. This inducible effect may be responding to different components of the ethyl acetate extract and depending on the age of walnut trees. The accumulation of allelochemicals in walnut trees are released into external environment (culture solution) from the roots to reduce the target plant growth. The reduction of the target plant growth occurred together with the inhibition of protective enzyme activity and root uptake ability, which is consistent with the previous studies (2,13,22,23,31).

## CONCLUSIONS

In *in-vitro* bioassay the root exudates of walnut trees (2,3, and 4 years old) inhibited the seed germination and seedling growth of turnip. Besides the ethyl acetate extract of root exudates of walnut trees also reduced the activities of protective enzyme systems and the content of malondialdehyde of turnip (*Brassica rapa* L.) three components (neutral, acidic, and alkaline). All results confirmed that the root exudates of walnut trees (2, 3, and 4 years old) showed a significant allelopathic effect and the allelopathic effect was stronger depending on the different components of root exudates of walnut trees and following the ages of walnut trees. The study in allelopathy effect of root exudates of walnut trees should be more specific. So, further studies are needed to identify the allelochemicals in root exudates of walnut trees, to get detailed information about the *in-vitro* allelopathic effect of root exudates of walnut trees.

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