

SHORT COMMUNICATION

Identification and bioassay of allelopathic substances from the root exudates of *Malus prunifolia*

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ABSTRACT

The root exudates of *Malus prunifolia* were collected and isolated using XAD-16 resin. The methanol extracts from the root exudates were detected with gas chromatography-mass spectroscopy (GC-MS). Twenty compounds (hydrocarbons, organic acid, phenol, esters, alcohol, benzene, etc.) were detected, but phthalate derivatives, butylated hydroxytoluene, sitosterol and palmitic acid occurred in higher quantity. The allelopathic effects of root exudates and four main identified compounds were determined on the radical elongation of *M. prunifolia*. The solutions of 4-compounds and root exudates inhibited the radical length of *M. prunifolia*. The degree of inhibition increased with the increasing concentration of root exudates and compounds. These results demonstrated that root exudates of *M. prunifolia* were autotoxic to its own seedling growth.

Key words: Autotoxicity, bioassays, exudates, growth inhibitors, identification, *Malus prunifolia*.

INTRODUCTION

Autotoxicity is a type of intraspecific allelopathy, where a plant specie inhibits the growth of its own kind through releasing toxic chemicals into the environment (21). This phenomenon has been widely reported in agroecosystems. Lettuce (*Lactuca sativa* L.), cucumber (*Cucumis sativus* L.), soybean [*Glycine max* (L.) Merr] and watermelon [*Citrullus lanatus* (Thumb.) Matsum and Nakai] causes autotoxic growth inhibition (10,15,17,18,33,34). The allelochemicals are released into the environment through leaching, root exudation, volatilization, or decomposition of plant residues (24). The root exudates represent the largest direct inputs of plant chemicals into the rhizosphere environment, therefore, they represent the largest source of allelochemicals (4). Much of the knowledge on autotoxicity in crops has resulted from the study of plant root exudates (2,29,30,34).

Apple (*Malus* sp.) is major fruit tree in China and its trees under go fruiting and senescence phases. Due to lack of new areas for planting, replanting has become a serious problem in fruit trees nurseries and old orchards, because replanting problem in apple

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orchards is not clearly understood. Previous studies have focused on the biotic factors, but the replanting problem occurs due to accumulation of soil-borne fungal, bacterial, nematodes and actinomycetes and pathogens causing diseases (16,19,27,28). Few studies have investigated the abiotic factors. Börner (5) attributed the apple replanting problem to the presence of chemical compounds in root bark being released into the orchard soil after microbial decomposition of fallen root bark. There are no recent published reports about the allelopathic potential of *M. prunifolia* root exudates collected and isolated using XAD-16 resin. Therefore, this study aimed to identify and evaluate the allelochemicals in the root exudates of *M. prunifolia*, so as to determine the autointoxication potential of *M. prunifolia* root exudates.

MATERIALS AND METHODS

Plant materials

Seeds of apple (*Malus prunifolia*) were obtained from Fuping County, Shaanxi Province on 28 August, 2007. Seeds were surface-sterilized in 0.3% (v/v) H₂O₂ for 20 min, followed by several rinses with sterile water on 23 December, 2007. The sterilized seeds were stratified in sand at 4 °C for 85 days. Sprouted seeds were sown in plastic pots (9 cm dia × 12 cm height; 3 seeds pot⁻¹) filled with sterilized sand. All pots were placed in greenhouse at Yangling (34°20'N, 108°24'E). The plants were grown without supplementary illumination at 20 to 25°C (Night/day temperature) and 65-80% relative humidity. Seedlings were irrigated once a week with Hoagland nutrient solution pH 6.0 ± 0.2 (11).

Collection and isolation of root exudates

Root exudates were prepared as per Tsanuo *et al.* (22) and Uddin *et al.* (25) with some modifications. When the seedlings reached 6-leaf stage, 80 seedlings were soaked in distilled water (500 ml) and kept at room temperature (25 °C). Only the roots of seedlings were immersed into the water. The distilled water was continuously aerated by air pumps. After 24 h, the aqueous extract was filtered through the filter paper (Whatman No.1) and a column (1.5 cm dia × 30 cm length) containing Amberlite XAD-16 resin (60 ml). The trapping column was eluted with 500 ml methanol at a flow rate of 4 ml min⁻¹. Then the eluates were concentrated with rotary evaporator at 37 °C (RE-3000 Rotary Evaporator; Shanghai Yarong Instruments Company, China) to a volume of 2 ml and stored at -20°C until use.

Bioassay

The bioassays were conducted in laboratory conditions with three replicates. The activity of suspected biologically active materials was evaluated in radical elongation tests using germinated seeds of *M. prunifolia*. The test solutions of root exudates (0.2, 0.4 or 0.8 ml with added distilled methanol to 2 ml) were added to sterilized 9 cm dia Petri dishes lined with filter paper (Whatman No.1), organic solvent was evaporated and dried remains was moistened with 5 ml distilled water. Ten germinated seeds were evenly placed on the filter paper in a Petri dish. Seeds on filter paper moistened only with 5 ml distilled water were used as control. Petri dishes with covers were sealed with parafilm and placed into

incubator (day/night regime of 12/12 h, 22/20 °C and the photon flux density of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Radical length was measured after 10 days. We did further bioassays following the same procedure with phthalic acid, sitosterol, butylated hydroxytoluene and palmitic acid (obtained from Yifang S&T Ltd. Tianjin, China). Each compound was diluted with sterile methanol to give final concentrations of 0.1, 1.0, 10, 20 mM.

Identification of allelopathic compounds

Trace GC 2000 (Polaris Q, USA) was used. The concentrated samples were filtered through 0.45 μm membrane filter then separated on a 30 cm \times 0.25 mm \times 0.25 mm capillary column. The GC-MS conditions were as follows: column at 50°C for 4 min, followed by 6°C/min to 250°C; injector at 250 °C; the injection volume was 1 μl ; ionization was set in the electron impact mode (EI) at 70 eV; mass spectra were repetitively scanned from 30 to 650 amu every 3 sec. The individual compounds were identified by comparing their mass spectra with those from NIST and Wiley Library of mass spectral database.

Statistical analysis: All bioassay data were subjected to analysis of variance (ANOVA) using Tukey's Studentised Range (HSD) Test (SAS Statistical package, version 8.2).

RESULTS AND DISCUSSION

All concentrations of root exudates inhibited the radical elongation of *M. prunifolia* seedlings (Table 1 and Fig. 1). The lower concentration (0.2 ml) of root exudates decreased the radical length of *M. prunifolia* by 6.48% than control and the decrement increased with increasing quantity of added solution compared to the control. At applied concentrations of 0.4 and 0.8 ml root exudates of *M. prunifolia*, the radical growth inhibition was 50.12 and 75.31%, respectively i.e. the inhibitory effects were enhanced with increasing concentrations of *M. prunifolia* root exudates. Allelopathy is a concentration-dependent phenomenon (8). Our findings agree with other studies. Similar trends have been observed in other crops (25,26). The methanol extracts of 8 plants inhibits the germination of maize (*Zea mays* L.) seeds (25). The different concentrations of *Albizia lebbek* leaf extract inhibits the germination and growth of crops (26).

Table 1. Effect of applied root exudates rates on radical growth of *M. prunifolia*

Amount added (ml)	Radical length (cm)
0	8.18 \pm 0.62 a
0.2	7.65 \pm 0.51 a
0.4	4.08 \pm 0.17 b
0.8	2.02 \pm 0.18 c

Means with the same letter are not significantly different ($P < 0.05$) by Tukey's studentized range test. Values are means of 3 replicates \pm S.E.

Resin is widely used to isolate and purify the allelochemicals from crops (13,14,33). The allelopathic substances from *M. prunifolia* root exudates trapped by XAD-16 resin, drastically inhibited the radical elongation of this specie. Our results are consistent with previous studies (13,14,33).

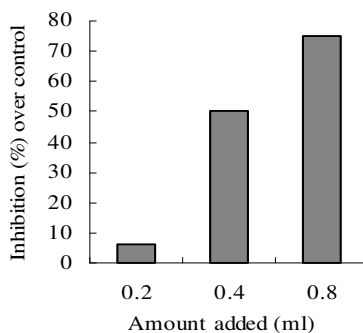


Figure 1. Inhibitory effects of root exudates application rates on the radical growth of *M. prunifolia*.

Analysis of root exudates with GC-MS resulted in the identification of > 20 components (Table 2). The root exudates mainly contained hydrocarbons, organic acid, phenols, esters, alcohol, benzene etc. In *M. prunifolia* root exudates, 4 compounds occurred in higher amounts [viz., phthalate derivates (52.49% peak area) and sitosterol (6.31%), followed by butylated hydroxytoluene (3.94%) and palmitic acid (3.81%)]. Phthalic acid, sitosterol, butylated hydroxytoluene and palmitic acid all inhibited the *M. prunifolia* seedlings radical growth (Table 3). All concentrations (0.1, 1.0, 10 and 20 mM) of 4 major compounds inhibited the radical growth of test seedlings. The inhibitory effects were enhanced with increasing concentrations of allelochemicals. Radical elongation of *M. prunifolia* was not significantly inhibited by 0.1 mM allelochemicals. At 20 mM phthalic acid, sitosterol, butylated hydroxytoluene and palmitic acid drastically inhibited the radical length by 81.36, 60.37, 75.85 and 62.29%, respectively (Fig. 2). The four major compounds analyzed by GC-MS in root exudates of *M. prunifolia* have been identified in agroecosystem and silviculture. Song *et al.* (22) identified phthalic acid in rice (*Oryza sativa*) root exudates at different phenological phases. Phthalic acid and derivatives were identified in rhizosphere-soil of rubber tree (*Hevea brasiliensis*) (31). Our results indicate that phthalic acid had allelopathic effects and are consistent with Chai *et al.* (6). Palmitic acid is a common phytotoxin and its toxic effects are known (12,15,20,32). Our results are consistent with them. Lee *et al.* (15) reported that the inhibition from palmitic acid was more as the concentration increased, the same trend was observed in our study. In this study, sitosterol inhibited the radical elongation in *M. prunifolia* seeds. Similar effects have been observed in *Typha latifolia* and *Eupatorium adenophorum* Spreng (1,3). The butylated hydroxytoluene inhibited the radical length of *M. prunifolia*. Butylated hydroxytoluene was identified in root exudates of *Zea mays* (6), *Cicer arietinum* (7) and *Glycine max* (9), however, its allelopathic effects are not reported.

These results indicated that autotoxic compounds were present in the root exudates of *M. prunifolia*, however, we evaluated only the major compounds present in higher amounts and the remaining needs to be evaluated. Their allelopathic effects can be additive, synergistic, or antagonistic (15,32). Thus, not only the compounds at higher

Table 2. Compounds isolated from the root exudates of *M. prunifolia* seedlings

Compound class	Compound name	Peak area (%)
Hydrocarbon	Hexadecane	1.09
	Octadecane	1.34
	Octacosane	1.84
	Tetracosane	1.49
	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	1.97
	Nonacosane	2.69
	Tetratetracontane	1.79
Organic acid	Palmitic acid	3.81
Phenol	Butylated hydroxytoluene	3.94
	Phenol, 2,4-bis(1-methylpropyl)-	0.84
	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	1.2
Ester	Phthalate derivatives	52.49
	Trilinolein	1.14
	9-Octadecenoic acid (Z)-, tetradecyl ester	1.82
	Stearic acid, 3-(octadecyloxy) propyl ester	2.72
	α -Sitosterol acetate	2.65
Alcohol	Sitosterol	6.31
Benzene	Ethylbenzene	1.19
	Benzene, 1,3-dimethyl-	2.93
	p-Xylene	1.29
Other	1H-Indene, 2-butyl-5-hexyloctahydro-	2.79
	Isoproturon	1.40
	Endrin	1.27

Table 3. Effects of levels of allelochemicals present in root exudates on radical growth of *M. prunifolia*

Allelochemicals	Concentration (mM)	Radical length (cm)
Control	0	3.81±0.43 a
Phthalic acid	0.1	3.68±0.28 a
	1.0	2.88±0.21 b
	10	1.12±0.27 de
	20	0.71±0.23 e
Sitosterol	0.1	3.77±0.40 a
	1.0	3.31±0.38 ab
	10	1.88±0.43 c
	20	1.51±0.35 cd
Butylated hydroxytoluene	0.1	3.71±0.49 a
	1.0	3.30±0.43 ab
	10	1.26±0.48 de
	20	0.92±0.09 de
Palmitic acid	0.1	3.71±0.33 a
	1.0	3.27±0.27 ab
	10	1.46±0.40 cd
	20	1.17±0.43 de

Means with the same letter are not significantly different ($P < 0.05$) by Tukey's studentized range test. Values are means of 3 replicates \pm S.E.

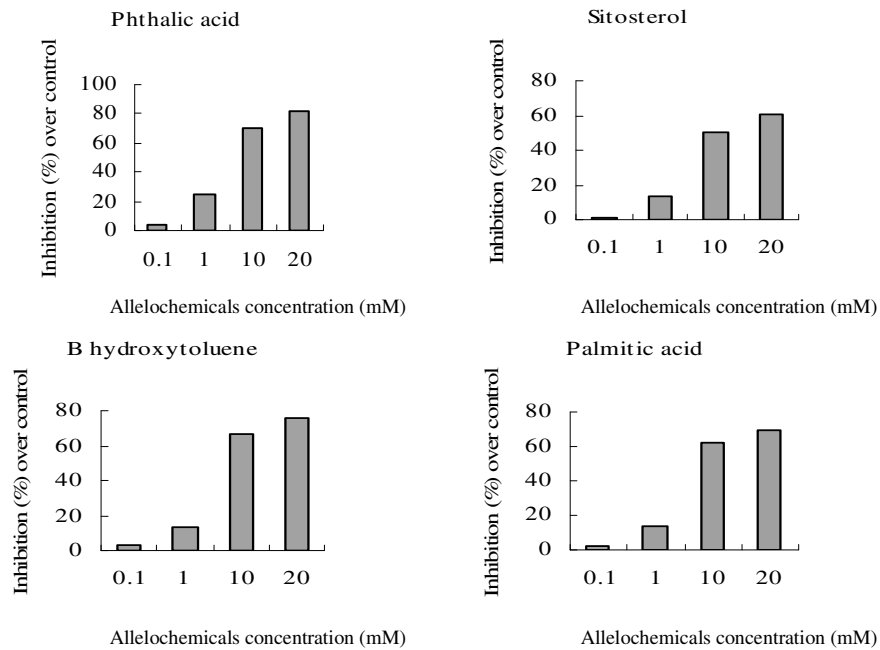


Figure 2. Inhibitory effects of allelochemicals applied rates on the radical growth of *M. prunifolia*.

concentrations but also compounds at lower ones are responsible for the allelopathic effects. It is important to examine the effects of mixed compounds on the growth of *M. prunifolia* plants. These problems remain to be solved.

CONCLUSIONS

We have demonstrated that root exudates of *M. prunifolia* reduced the radical growth of own seedlings, due to secondary metabolites. Allelopathic substances from *M. prunifolia* root exudates drastically inhibited the radical elongation of *M. prunifolia*. This study may help in understanding the allelopathic potential of *M. prunifolia* and may provide more information about the abiotic factors related to the replanting problem in apple orchards.

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