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Effects of arbuscular mycorrhizal fungi inoculation on allelopathic potential of *Artemisia annua* L. root exudates

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ABSTRACT

We investigated the effects of residual allelochemicals in crop rotation on the succeeding crops (radish, cucumber and ryegrass) sown after *Artemisia annua* L. The arbuscular mycorrhizal fungi (AMF) *Glomus mosseae* and without inoculation and sown in pots to collect root exudates from hydroponic solution and matrix by extractives and direct concentration. The main component of *A. annua* L. root exudates was identified by gas chromatography-mass spectrometry (GC-MS). Then, radish (*Raphanus sativus* L.), ryegrass (*Lolium perenne* L.) and cucumber (*Cucumis sativus* L.) were sown as recipient plants for the allelopathic bioassay of root exudates. The main component of *A. annua* L. root exudates was dibutyl phthalate. The allelopathic bioassay showed inhibition of germination rate (GR), germination potential (GE), germination index (GI), shoot height, root length, shoot dry weight (DW) and root dry weight (DW) of recipient plants. The inhibitory effects of root exudates were more remarkable on ryegrass than on radish and cucumber. *Artemisia annua* L. root exudates residues inhibited the seed germination and seedlings growth of subsequent crops. This indicated that *Artemisia annua* L. might not be suitable for rotation with radish, cucumber and ryegrass. Compared with non-mycorrhizal (NM) plant, mycorrhizal (AM) plant enhanced the allelopathy effects of *Artemisia annua* L., with greater secretion of root exudates. Besides, the root exudates obtained by different methods had variable allelopathic effects. For example, root exudates collected by extractive concentrate mrthod were more inhibitory than collected by direct concentrate, i.e. the root exudates were easily extracted by ethyl acetate. The root exudates collected from matrix were more inhibitory to recipient plants than those collected from hydroponic solution, indicating that more root exudates were retained in the matrix.

Key words: Allelopathic bioassay, Allelopathy, Arbuscular mycorrhizal fungi, *Artemisia annu*a, cucumber, *Cucumis sativus,* GC-MS, *Glomus mosseae,* hydroponics, *Lolium perenne,* matrix, radish, *Raphanus sativu*, residual allelochemicals, root exudates, ryegrass, germination, seedlings growth.

INTRODUCTION

Plants affect themselves or their surroundings plants through release of allelochemicals into environment. More than 100,000 allelochemicals are released by plants (13). In nature, plant allelopathy affects the intraspecific or interspecific relationships, community succession, species distribution pattern and biological invasion (10,33). Allelopathy plays role in crop cultivation and may lead to development of new ecofriendly pesticides (7,16,20). Compared with single compound, synergism between the allelochemicals produces stronger effects on recipient organisms and these compounds have advantages of easy extraction, high activity and simple structure (19).

Arbuscular mycorrhizal fungi (AMF) develops mutually beneficial symbiotic association with > 80% plants (29,31). After inoculating the host root, AMF absorbs

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nutrients from plants to support own growth, besides, the AMF also helps the plants to absorb nutrients from soil and improve the activity of root exudates. Thus the plants show better growth and improved resistance to stresses. (14,22). AMF (*Glomus mosseae*, *Glomus versiforme* and *Glomus etunicatum*) promotes the growth of grapes, activity of superoxide dismutase (SOD) and organic acids and decreased the malondialdehyde (MDA) content (11). *Glomus mosseae* had significant positive effects on the host plants. Stefanowicz *et al.* (25) reported that the invasion of *Rosa rubra* L. was accompanied by increase in AMF biomass and also increased the secondary metabolites secreted by plant roots. Chen *et al.* (4) reported that AMF (*Glomus mosseae*) inoculation improves the root system architecture, flavonoids accumulation and uptake of P, K, Mg, Cu, Zn and Mn of liquorice under nutrients stress. Chen *et al.* (6) proved that AMF (*Glomus mosseae* and *Glomus versiforme*) promoted the growth and pathogenic defence of *Wedelia trilobata* L. in nutrients-poor environment, which may help in successful invasion into certain type of habitats. The flavonoids and strigolactones in root exudates work as signal molecules in symbiotic and pathogenic plant-fungus interactions (26). AMF might increase the root exudates of host plants and then cause allelopathic inhibition on surrounding organisms, which is conducive to resource acquisition for themselves (12). In our previous studies, we have found that various AMF species have different colonization rates on the *Artemisia annua* L. roots. The colonization rate of *Artemisia annua* L. roots inoculated with *Glomus mosseae* was higher than with *Glomus versiforme*, thereby plants absorb more N P K, leading to increased photosynthesis and higher artemisinin content (12). Thus, *Glomus mosseae* was selected for the present study.

Figure 1. Photograph of donor specie *Armtemisia annua* L. plant

Artemisia annua L. (Figure 1) plant has detoxification effects. In 1970s, Artemisinin was successfully extracted and isolated from *A. annua* (9). It proved most effective drug for malaria and also have anti-tumor properties (15). Chen *et al.* (5) reported that *A. annua* showed strong invasion by releasing the allelochemicals to inhibit the growth of adjacent plants. Duke *et al.* (8) found that artemisinin inhibited the growth of weeds at 33 µmol/L concentration. Lyden *et al.* (18) reported that not only artemisinin, but also water-soluble and fat-soluble extracts of *A. annua* inhibited the growth of surrounding

plants. Mu *et al.* (20) found that *A. annua* root exudates collected in hydroponic solution inhibited the growth 9of wheat seedlings and the inhibition increased with increasing concentrations in hydroponic solution. However, the allelopathy of *A. annua* with AMF inoculation in roots is still not well explored.

Crop rotation is common farming practice, which means that different crops are sown in turn one after the other in the same field within a year. To reduce the adverse effects of continuous cropping problems on crops, Radish (*Raphanus sativus* L.), cucumber (*Cucumis sativus* L.) and ryegrass (*Lolium perenne* L.) are grown in crop rotations in southern China. Radish is winter vegetable in Guangxi and ryegrass is winter green manure or forage. The affects of allelochemical from crop residues of *Artemisia annua* L. on subsequent crops (radish, cucumber and ryegrass) needs to be studied. The radish, cucumber and ryegrass are also allelopathic indicators sensitive to allelochemicals, hence selected as recipient plants. This study aimed to investigate the effects of root exudates of *Artemisia annua* L. with and without AMF inoculation and their allelopathic effects on recipient plants (radish, cucumber and ryegrass).

MATERIALS AND METHODS

This study was conducted in greenhouse of Guangxi University in Nanning (N 22°50', E 108°17'), China. Nanning has subtropical monsoon climate, mean annual rainfall: 1304 mm, mean annual temperature: 22 ºC and mean height above sea level: 96 m. Day length: 12 h. The experiment began on May 02, 2014. *Artemisia annua* L. plants were grown for 4-months in greenhouse [temperatures 29/25ºC (day/night) and humidity of 79%].

Plant materials, mycorrhizal fungus and growth matrix: The seed and plant material of *Artemisia annua* L. (Figure 1) Youqing No. 1 were obtained from Chongqing Fumin Qinghao Technology Co., Ltd. The arbuscular mycorrhizal fungus *Glomus mosseae* (No. NM02A) was obtained from Bank of Glomeromycota in China (BGC). Sand-based mycorrhizal inoculum contains 120 propagules g^{-1}, consisting of spores, hyphae and colonial roots. Radish (*Raphanus sativus* cv Yumanchun L.), ryegrass (*Lolium perenne* cv Fuyuan) and cucumber (*Cucumis sativus* L.) were the recipient plants in allelopathic bioassay. The seeds were obtained from the local agricultural market. Growth matrix: Sand (obtained from the local building materials market), peat (obtained from Pindstrup Mosebrug A/S, Denmark).

The experimental treatments consisted of 3 factors: (I). Inoculation: (i). AMF inoculated, (ii). Not inoculated, (II). Methods of root exudates collection: (i). Fom substrate (ii). From hydroponics), (III). Methods of root exudates concentration: (i). Extraction (ii). direct concentration).

Mycorrhiza inoculum and growing conditions: *Artemisia annua* L. was inoculated and not inoculated with arbuscular mycorrhizal fungi (AMF) and sown in pots in greenhouse. Each treatment was replicated twenty times. The pots were filled with sand and peat in 4:1 (w/w). The sand was sieved (< 3 mm) to remove stones and then washed by tap water. After drying, the mixture was autoclaved (1 h at 121 ºC) prior to use and used at 3 kg per pot. The inoculation treatment group was inoculated with 150 g inoculum (Sand-based mycorrhizal inoculum (spores, hyphae) contained 120 propagules g^{-1}, per pot and layered 2

cm below the substrate surface. The Non-mycorrhizal treatments, received similar inoculum per pot (autoclaved for 1 h at 121°C). The seeds of *A. annua*. were surface-sterilized by immersion for 2 min in 10 % hydrogen peroxide (v/v), rinsed thrice in distilled water and dried at room temperature. Five disinfected *A. annua* seeds per pot were sown 2 cm deep in plots and irrigated. Hoagland nutrient solution (Table 1) @ 200 mL per pot, was applied twice a week after the seedlings emergence and irrigated as needed.

Table 1. The composition of Hoagland nutrient solution

Components	Concentration ($mg \cdot L^{-1}$)
KH_2PO_4	136
KNO_3	506
$Ca(NO_3)_2 \cdot 4H_2O$	1180
$MgSO_4 \cdot 7H_2O$	493
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.08
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.09
Fe-EDTA $\{^{EDTA.Na}_{FeSO_4 \cdot 7H_2O}\}$	7.45 5.57

Mycorrhizal colonization rate: After 4 months, *A. annua* roots were randomly sampled 5-times per pot with 1 cm dia puncher [(cleaned in 5 % KOH (w/v) for 30 min at 90 ºC)]. After rinsing with tap water, the samples were acidified in 2 M HCl for 5 min and then stained with 0.01 % fuchsin in 90 % lactic acid solution for 20 min at 90 ºC. Colonization was quantified from 200 root segments under a dissecting microscope with 40× magnification (17).

Root exudates collection: After 4 months, *A. annua* root exudates were collected from hydroponic solution and matrix respectively.

(i). **Collection from hydroponic solution:** The whole plants were sampled from the pots and the matrix remaining on the roots was washed with tap water without damaging the plants. These were brought to the laboratory in ice boxes and the roots were washed with distilled water thrice. Surface water droplets were absorbed with absorbent papers. The plants roots were placed in 0.5 mmol/L $CaCl_2$ solution for 30 min and then transferred to 1.7 L shade containers containing 250 mL $CaCl_2$ solution (0.5 mmol/L). Roots were immersed in $CaCl_2$ solution. Plants were grown under sufficient light for 4 h and then removed from the containers. Plant roots were washed thrice with distilled water. The flushing solution and $CaCl_2$ solution of hydroponically cultured plants were combined to form root exudates of hydroponic solution. The solution was filtered through 0.22 μm membrane by vacuum filtration and then stored at -20 ºC until used.

(ii). **Collection from matrix:** Remove the whole plants and the residual roots from the matrix. Fifty g matrix was transferred to 150 mL conical flask and 100 mL distilled water was added to the flask to extract root exudates by oscillation for 24 h. The solution was filtered through double-layer filter paper and then filtered through 0.22 μm membrane by vacuum filtration and then stored at -20ºC until used.

Root exudates concentration: The *A. annua* root exudates were concentrated by following two methods.

(i). **Extractive concentrate:** The root exudates solution was extracted thrice by ethyl acetate. The ratio of ethyl acetate and root exudates solution was 1:1 (v/v). Ethyl acetate layer was concentrated by rotary evaporator, then (i) dissolved in 0.5 mL methanol to identify the main components of *A. annua* root exudates and (ii) dissolved in 20 mL methanol for allelopathic bioassay.

(ii). **Direct concentrate:** The root exudates solution was directly concentrated by rotary evaporator and was dissolved in 20 mL distilled water for allelopathic bioassay.

Compounds Identification

The extractive concentrate of *Artemisia annua* L. root exudates were investigated using gas chromatography-mass spectrometry (GC-MS) on a Thermo Scientific Finnigan Trace GC Ultra DSQ-II(Thermo Scientific, San Jose, USA) in Guangxi Analytical and Testing Center. The system was equipped with a TR-5MS capillary column (30 m×0.25 mm inner diameter, 0.25 μm film thickness). The temperatures of ion source, injection and transfer line were 230 ºC, 250 ºC and 280 ºC, respectively. Column temperature was kept at 60 ºC for 2 min, then increased to 140 ºC @ 10 ºC/min, finally to 280 ºC @ 5 ºC/min for 6 min. A 1 μL aliquot of analyte was injected into GC, which was run in splitless model, using helium as the carrier gas at flow rate of 1.0 mL/min. The MS data were acquired in full-scan mode with the mass-to-change (m/z) ratio range of 35-650 amu after a solvent delay of 6 min. Xcalibur software (Thermo Scientific, San Jose, USA) was used for identifying the main components of *A. annua* root exudates.

Bioassay

Seed germination: Radish (*Raphanus sativus* L.), ryegrass (*Lolium perenne* L.) and cucumber (*Cucumis sativus* L.) seeds were surface-sterilized by immersing for 2 min in 10% (v/v) hydrogen peroxide, rinsed thrice in distilled water and dried at room temperature.

Twenty seeds of each test plant were kept in separate beaker (100 mL). Distilled water was used as control. Each treatment was replicated thrice. Two mL aliquot of test root exudates solution (extractive concentrate) was added to the filter paper as under:

(i). Root exudates of hydroponic solution of *A. annua* without AMF inoculation (NM-WE),

(ii). Root exudates of hydroponic solution of *A. annua* with AMF inoculation (AM-WE),

(iii). Root exudates of matrix of *A. annua* without AMF inoculation (NM-SE),

(iv). Root exudates of matrix of *A. annua* with AMF inoculation (AM-SE).

Methanol was used as control. After complete evaporation of methanol in beaker, 2 mL distilled water was added.

At the same time, another 2 mL aliquot of test root exudates solution (direct concentrate) was added to the filter paper as under :

(i). Root exudates from hydroponic solution of *A. annua* without AMF inoculation (NM-WD),

(ii). Root exudates from hydroponic solution of *A. annua* with AMF inoculation (AM-WD),

(iii). Root exudates from matrix of *A. annua* without AMF inoculation (NM-SD),

(iv). Root exudates obtained by direct concentrate from matrix of *A. annua* with AMF inoculation (AM-SD).

The beakers were kept in incubator (25ºC, 12 h light) and 2 mL distilled water was added daily to each beaker. The seed germination rate (GR), germination potential (GE) and germination index (GI) of recipient plants were calculated as under.

GR (%) = Number of germinated seeds at the end of germination/Number of seeds tested) × 100%;

GE (%) = Number of germinated seeds 7 days/Number of seeds tested) × 100%;

GI = Σ (Number of seeds germinated on t day after sowing/t).

Seedling growth

On the 7th day, height of recipient plants seedlings was measured. Plants were uprooted and gently washed to preserve the roots. Epson Expression 11000XL Scanner was used to scan the roots and WinRHIZO software (Regent Instruments Canada Inc., Quebec, Canada) was used to analyze the root length. The shoots and roots were oven-dried at 70ºC for 48 h for dry weight (DW).

Statistical analysis

Statistical differences in seed germination and seedling growth index for 3 recipient plants amongst different treatments were analyzed by one-way analysis of variance (ANOVA) using the R-3.6.0 (R Core Team, 2019). To compare the level of allelopathy, the significance of stimulation or inhibition was determined using the allelopathic response index (RI) designed by Williamson and Richardson (30).

$$RI = \begin{cases} 1 - C/T, & T \geq C \\ T/C - 1, & T < C \end{cases}$$

Where, T: Seed germination or seedling growth response of recipient plants treated with root exudates and

C: Seed germination or seedling growth response of recipient plants in control.

Positive RI value indicates that *A. annua* root exudates stimulated the seed germination or seedling growth, whereas, a negative RI value indicates that the root exudates inhibited the seed germination or seedling growth. The absolute value of RI reflects the intensity of allelopathy.

Allelopathic synthesis effects: It is the arithmetic mean value of RI of all physiological indices, which reflects the comprehensive effects of root exudates on recipient plants. A positive synthesis effect value indicates that the *A. annua* root exudates were beneficial to recipient plants, whereas a negative synthesis effect value indicates that the root exudates were harmful to recipient plants.

RESULTS AND DISCUSSION

I. Mycorrhizal colonization rate

At the end of the experimental period (4-months) no mycorrhizal colonization was observed in the roots of non-inoculated *A. annua*. However, the mycorrhizal colonization rate in the roots of mycorrhizal inoculated *A. annua* was 52.8% and the mycelia and typical vesicle structure in the roots were clearly observed under the microscope (Figure 2).

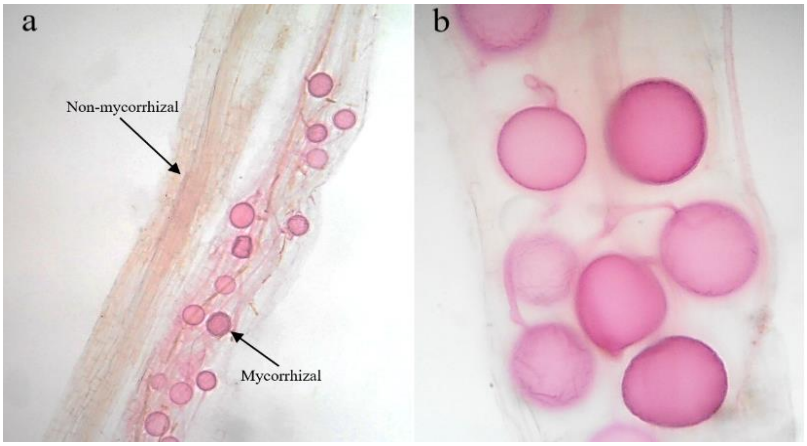

Figure 2 **a**. Morphology of mycorrhizal and non-mycorrhizal root of *Artemisia annua* L.; **b** Morphology of hyphae and vesicles in the mycorrhizal root

In our previous study, Huang *et al.* (12) AMF (*Glomus mosseae*) colonized the roots of *A. annua*. After four months of cultivation, the mycorrhizal colonization rate was 62.9%. Among other plants, Guo *et al.* (11) reported that the colonization rate of *Glomus mosseae* on grapes was 52.5%, much higher than *Glomus versiforme* and *Glomus etunicatum*. Chen *et al.* (4) found that among the liquorice plants treated with *Glomus mosseae*, 85% were successfully colonized by *Glomus mosseae*. In this study, the mycorrhizal colonization rate was 52.8%, which was similar to previous studies. The high level of mycorrhizal colonization rate indicated that *A. annua* and AMF (*Glomus mosseae*) can form a stable symbiotic relationship.

II. *Artemisia annua* L. root exudates components

The effects of collection method and AMF of the chemical composition of *A. annua* root exudates was analyzed by GC-MS (Table 2). Except the simple alkanes, there were 18 compounds in NM-W, 23 compounds in AM-W, 19 compounds in NM-S and 18 compounds in AM-W. These compounds mainly included phenolic compounds, terpenoids, allelochemicals containing a nitrogen atom, alkane ketone, fatty acids, water-soluble organic acids and other substances. Phthalic acid esters (didodecyl phthalate, butyl cyclohexyl phthalate, diisobutyl phthalate, dibutyl phthalate and di-n-heptyl phthalate) were the main components of *Artemisia annua* L. root exudates and the relative content of dibutyl phthalate was the highest. The relative content of phthalate esters in matrix was higher than in hydroponic solution. AMF symbiosis increased the relative content of phthalate esters in root exudates.

Simple phenols and their derivatives are allelochemicals (19) and the biological activity of dibutyl phthalate has been reported (22). AMF symbiosis increased the content of dibutyl phthalate in root exudates, which might, have enhanced the allelopathy of plants. AMF promotes the root growth and morphology of plants (4), thereby secreting more allelochemicals. The plants growth was promoted, which helped the AMF to obtain

Table 2. Effects of collection methods and AMF on chemical components of *A. annua* root exudates

Compounds	Molecular formula	Relative content (%)			
		NM-W	AM-W	NM-S	AM-S
Phenolic compounds					
Didodecyl phthalate	$C_{32}H_{54}O_4$	0.22	0.21	0.48	1.83
Butyl cyclohexyl phthalate	$C_{18}H_{24}O_4$	2.31	9.75	—	—
Diisobutyl phthalate	$C_{16}H_{22}O_4$	—	—	12.65	16.55
Dibutyl phthalate	$C_{16}H_{22}O_4$	—	—	14.94	18.12
Di-n-heptyl phthalate	$C_{22}H_{34}O_4$	—	—	1.93	1.54
4-Aminobenzoic acid	$C_7H_7NO_2$	—	4.22	—	—
4-Ethylresorcinol	$C_8H_{10}O_2$	—	0.88	—	—
Thymol	$C_{10}H_{14}O$	3.29	0.21	—	—
2-Methoxy-4-propylphenol	$C_{10}H_{14}O_2$	—	7.36	—	—
2-Methoxy-4-(1-propenyl)phenol	$C_{10}H_{12}O_2$	3.31	3.45	—	—
Methyl 2-furoate	$C_6H_6O_3$	—	0.31	—	—
3,5-Di-t-butyl-1,2-benzoquinone	$C_{14}H_{20}O_2$	1.19	—	1.04	1.07
Terpenoids					
Isoterpinene	$C_{10}H_{16}$	0.48	0.64	—	—
Alpha-terpinen	$C_{10}H_{16}$	—	0.80	—	—
Allelochemicals containing nitrogen atom					
1,2,4,5-Tetrazine	$C_2H_2N_4$	—	—	0.37	0.91
Pyrrole	C_4H_5N	—	—	0.6	0.44
Pyrrolidine	C_4H_9N	—	—	—	0.24
2-(4-Methylphenyl)pyridine	$C_{12}H_{11}N$	2.21	0.88	—	—
2-Methyl-5-acetoxy pyridine	$C_8H_9NO_2$	9.29	4.51	—	—
Ethylenimine	C_2H_5N	—	—	—	2.31
1-Butanamine	$C_4H_{11}N$	—	—	1.14	1.52
p-Phenylenediamine	$C_{10}H_{16}N_2$	2.43	—	—	—
Octodrine	$C_8H_{19}N$	—	—	1.06	—
Formic acid hydrazide	CH_4N_2O	—	—	0.41	0.28
Alkane ketone					
Isolongifolanone	$C_{15}H_{24}O$	6.48	7.97	—	—
Fatty acids					
N-hexadecanoic acid	$C_{16}H_{32}O_2$	—	6.77	—	—
Polyethylene glycol monostearate	$C_{22}H_{44}O_4$	—	1.59	—	—
Water-soluble organic acids					
Acetic anhydride	$C_4H_6O_3$	—	0.30	—	—
Butanoic acid	$C_4H_8O_2$	0.29	—	—	—
Azelaic acid	$C_9H_{16}O_4$	—	0.18	—	—
Pentanoic acid	$C_5H_{10}O_2$	0.20	—	—	—
Gibberellic acid	$C_{19}H_{22}O_6$	—	0.58	—	—
Others					
2-Methoxy-1,3-dioxolane	$C_4H_8O_3$	—	—	—	1.01
Methylnitrite	CH_3NO_2	0.20	0.19	0.20	0.17
Acetic acid, oxo-methyl ester	$C_3H_4O_3$	—	—	0.48	0.72
2-Hydroxyethyl formate	$C_3H_6O_3$	0.34	—	0.13	—
Glycerine	$C_3H_8O_3$	15.33	3.63	0.28	0.19
3-Methyl-3-pentanol	$C_6H_{14}O$	—	1.92	—	—
(Z)-11-Hexadecen-1-ol	$C_{16}H_{32}O$	5.02	—	1.74	0.43
(E)-9-Tetradecen-1-ol	$C_{14}H_{28}O$	—	—	0.89	—
3-methyl-5-Hexen-3-ol	$C_7H_{14}O$	2.61	—	1.13	—
Cyclopentadecanol	$C_{15}H_{30}O$	—	3.97	—	—
6-cis-Vitamin A aldehyde	$C_{20}H_{28}O$	1.35	0.57	0.91	—
Undecanal	$C_{11}H_{22}O$	—	—	1.3	1.19
3-Furaldehyde	$C_5H_4O_2$	—	—	—	1.07

NM-W: Root exudates obtained from hydroponic solution of *A. annua* without AMF inoculation;
AM-W: Root exudates obtained from hydroponic solution of *A. annua* with AMF inoculation;
NM-S: Root exudates obtained from matrix of *A. annua* without AMF inoculation;
AM-S: Root exudates obtained from matrix of *A. annua* with AMF inoculation

nutrients from plants. Secretion of allelochemicals may be beneficial to plant-fungus symbiosis system. In addition, dibutyl phthalate was also detected in the matrix, but not in the hydroponic solution, indicating that dibutyl phthalate mainly remained in the matrix. It proved the presence of dibutyl phthalate in the root exudates.

III. Seed germination

The allelopathic effects of root exudates obtained by extractive concentrate on seed germination 3-recipient plants are presented in Table 3. NM-WE significantly inhibited the radish seeds germination potential and germination index than control. NM-SE and AM-SE were also significantly inhibitory to ryegrass seeds germination potential energy. AM-SE also significantly inhibited the germination of cucumber seeds.

Table 3. Allelopathic effects of root exudates obtained by extractive concentrate method and AMF on seed germination of 3- recipient plants (radish, ryegrass and cucumber).

Treatment	Germination rate (%)	Germination potential (%)	Germination index
Radish			
Control	100.00±0.00a	93.33±5.77a	19.22±0.90a
NM-WE	100.00±0.00a	73.33±2.89b	16.95±0.16b
AM-WE	100.00±0.00a	81.67±7.64ab	18.11±0.90ab
NM-SE	100.00±0.00a	86.67±5.77ab	18.56±0.68ab
AM-SE	98.33±2.89a	86.67±2.89ab	18.50±0.48ab
Ryegrass			
Control	90.00±8.66a	83.33±5.77a	7.17±0.31a
NM-WE	88.33±2.89a	88.33±7.63a	7.28±0.30a
AM-WE	88.33±5.77a	83.33±2.89a	6.56±0.22a
NM-SE	90.00±0.00a	51.33±2.89b	6.50±0.19a
AM-SE	88.33±2.89a	51.33±8.66b	6.44±0.16a
Cucumber			
Control	100.00±0.00a	88.33±2.89a	12.06±0.47a
NM-WE	100.00±0.00a	93.33±5.77a	11.56±0.23a
AM-WE	100.00±0.00a	93.33±2.89a	11.33±1.14a
NM-SE	100.00±0.00a	98.33±2.89a	13.11±1.05a
AM-SE	93.33±2.89b	90.00±5.00a	11.39±0.67a

Values are means ±SD, n=3; Different small letters indicate significant difference between the treatments (LSD test, $P<0.05$);
NM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* without AMF inoculation; AM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* without AMF inoculation; AM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* with AMF inoculation

The allelopathic effects of root exudates obtained by direct concentrate method on seed germination of 3-recipient plants are given in Table 4. AM-SD significantly stimulated the radish seeds germination energy over control. While, AM-SD significantly inhibited the rye grass seeds germination rate and germination index. Likewise, the AM-WD, NM-SD and AM-SD significantly inhibited the cucumber seeds germination index.

Seed germination is an important step and crucial in plants survival (1,18), which mainly depends on suitable environmental conditions (28). Root exudates released into the solution, affects the seeds germination of recipient plants. The accumulation of root exudates and their potential role in plant-plant interactions is known for several crops and medicinal plants (16). Our study found that only direct concentrate of AM-SD in low amount significantly promoted the radish seeds germination, which might be due to the breakdown of seed dormancy. The root exudates inhibited the seeds germination of three recipient plants (radish, cucumber and ryegrass). *Artemisia annua* L. has an advantage over other plants in competition due to inhibition of seed germination. However, this phenomenon adversely effects the seed germination of subsequent crops.

Table 4. Allelopathic effects of root exudates obtained by direct concentrate method and AMF on seed germination of 3- recipient plants (radish, ryegrass and cucumber).

Treatment	Germination rate (%)	Germination potential (%)	Germination index
Radish			
Control	100.00±0.00a	86.67±5.88bc	18.11±1.28a
NM-WD	100.00±0.00a	95.00±0.00ab	19.28±1.14a
AM-WD	98.33±5.77a	85.00±2.89c	18.00±1.93a
NM-SD	100.00±0.00a	81.67±5.77c	17.83±1.57a
AM-SD	100.00±0.00a	96.67±2.89a	19.50±1.96a
Ryegrass			
Control	95.00±0.00a	76.67±2.89a	6.44±0.44a
NM-WD	93.33±2.89a	78.33±5.77a	6.84±0.53a
AM-WD	93.33±2.89a	73.33±2.89a	6.22±0.27a
NM-SD	91.67±8.66a	71.67±2.89a	6.11±0.36a
AM-SD	85.00±2.89b	71.67±2.89a	5.72±0.16b
Cucumber			
Control	91.67±0.00a	90.00±5.00a	14.06±1.13a
NM-WD	90.00±2.89a	88.33±2.89a	13.23±0.88ab
AM-WD	93.33±2.89a	83.33±5.77a	12.56±1.28cd
NM-SD	96.67±2.89a	85.33±5.77a	11.75±0.93d
AM-SD	93.33±4.50a	86.67±5.00a	12.78±1.31c

NM-WD: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* without AMF inoculation; AM-WD: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SD: Root exudates obtained by extractive concentrate from matrix of *A. annua* without AMF inoculation; AM-SD: Root exudates obtained by extractive concentrate from matrix of *A. annua* with AMF inoculation.

IV. Seedling growth

The allelopathic effects of root exudates obtained by extractive concentrate method on seedlings growth of 3-recipient plants are given in Table-5. In radish seedlings, AM-WE significantly inhibited the shoot height and root length over control, while, NM-SE significantly inhibited the shoot height and AM-SE significantly inhibited the shoot height, root length and shoot Dry weight. In ryegrass seedlings, NM-WE significantly inhibited shoot Dry weight; AM-SE markedly inhibited the shoot height, shoot Dry weight. For cucumber seedlings, AM-WE significantly inhibited the root Dry weight.

Table 6 showed the allelopathic effects of root exudates obtained by direct concentrate method on seedling growth of 3-recipient plants. In comparison to control, NM-WD significantly inhibited the root length, but promoted the root Dry weight; AM-WD and NM-SD significantly inhibited the root length; AM-SD significantly inhibited the root length in radish seedlings. For ryegrass seedlings, NM-WD significantly inhibited the shoot Dry weight; NM-SD significantly inhibited the root length; while, the AM-SD significantly inhibited the root length, shoot Dry weight and root Dry weight. For cucumber seedlings, NM-WD significantly inhibited the shoot height. NM-SD significantly inhibited the root length; AM-SD significantly inhibited the root length, shoot Dry weight.

Table 5. Allelopathic effects of root exudates obtained by extractive concentrate method and AMF on seedlings growth of 3- recipient plants (radish, ryegrass and cucumber)

Treatment	Shoot length (cm)	Root length (cm)	Shoot DW (g/plant)	Root DW (g/plant)
Radish				
Control	6.41±0.46a	11.62±1.08a	0.210±0.012a	0.028±0.002ab
NM-WE	5.38±0.29ab	9.43±0.37ab	0.201±0.033a	0.032±0.001ab
AM-WE	4.54±0.21b	7.31±0.37b	0.192±0.013a	0.024±0.002b
NM-SE	4.86±0.55b	10.41±0.46ab	0.210±0.109a	0.040±0.005a
AM-SE	4.50±0.21b	9.71±1.25ab	0.179±0.022b	0.026±0.005ab
Ryegrass				
Control	7.13±0.79a	5.08±0.47a	0.025±0.003a	0.065±0.006ab
NM-WE	5.89±0.39ab	5.44±0.37a	0.018±0.002b	0.059±0.005b
AM-WE	6.59±0.20ab	5.61±0.26a	0.023±0.000ab	0.061±0.003b
NM-SE	6.74±0.17ab	5.94±1.20a	0.023±0.002ab	0.071±0.008a
AM-SE	4.54±0.24b	5.33±0.29a	0.017±0.001b	0.061±0.003b
Cucumber				
Control	3.94±0.16a	21.04±1.57a	0.353±0.018a	0.087±0.008a
NM-WE	3.98±0.21a	20.78±1.06a	0.344±0.012a	0.088±0.003a
AM-WE	4.02±0.22a	19.84±1.31a	0.342±0.019a	0.079±0.007ab
NM-SE	3.52±0.26a	18.81±1.13a	0.363±0.019a	0.089±0.007a
AM-SE	4.15±0.17a	18.29±1.30a	0.330±0.018a	0.070±0.007b

Values are means ±SD, n=3; Different small letters indicate a significant difference between the treatments (LSD test, $P<0.05$);

NM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* without AMF inoculation; AM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* without AMF inoculation; AM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* with AMF inoculation.

A. annua is rich in sesquiterpene lactones (20). These sesquiterpene lactone contains the endoperoxide moiety ans has strong antimalarial activity (32) and inhibits the surrounding plants growth (2). Both effects are related to the endoperoxide moiety, because deoxyartemisinin has no activity (20). The root exudates of *A. annua* had different effects on 3-recipient plants (radish, ryegrass and cucumber) seedlings. Most treatments inhibited the growth of recipient plant seedlings. As the "novel weapons hypothesis" (3), *A. annua* inhibits the growth of recipient plant seedlings by producing root exudates, which had more advantages in resource competition (24). This phenomenon is harmful to the

seedling growth of subsequent crops, indicating that *A. annua* is not suitable for rotation with radish, cucumber and ryegrass.

Table 6. Allelopathic effects of root exudates obtained by direct concentrate method and AMF on seedlings growth of 3- recipient plants (radish, ryegrass and cucumber)

Treatment	Shoot length (cm)	Root length (cm)	Shoot DW (g/plant)	Root DW (g/plant)
Radish				
Control	4.80±0.46a	15.00±1.21a	0.221±0.010a	0.034±0.003b
NM-WD	4.60±0.21a	13.62±0.73b	0.211±0.031a	0.044±0.002a
AM-WD	4.69±0.27a	11.20±0.66b	0.221±0.013a	0.030±0.002b
NM-SD	5.23±0.75a	7.29±1.28c	0.221±0.103a	0.033±0.005b
AM-SD	4.90±0.28a	12.36±0.32b	0.210±0.022a	0.026±0.005b
Ryegrass				
Control	3.10±0.19a	5.46±0.47a	0.020±0.003a	0.059±0.007a
NM-WD	3.45±0.20a	5.23±0.26a	0.021±0.001a	0.060±0.003a
AM-WD	3.26±0.21a	5.00±0.37a	0.022±0.002a	0.050±0.005ab
NM-SD	3.36±0.21a	4.91±0.25b	0.019±0.002a	0.057±0.011a
AM-SD	3.53±0.48a	4.11±0.29c	0.017±0.001b	0.044±0.003b
Cucumber				
Control	6.49±0.58a	27.46±1.71a	0.332±0.018a	0.103±0.009a
NM-WD	5.91±0.27b	25.34±1.06a	0.301±0.002ab	0.115±0.003a
AM-WD	6.30±0.41ab	25.80±1.14a	0.300±0.019ab	0.098±0.006a
NM-SD	6.27±0.19ab	23.89±1.14b	0.300±0.011ab	0.120±0.007a
AM-SD	6.13±0.25ab	23.78±1.38b	0.283±0.018b	0.094±0.007a

NM-WD: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* without AMF inoculation; AM-WD: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SD: Root exudates obtained by extractive concentrate from matrix of *A. annua* without AMF inoculation; AM-SD: Root exudates obtained by extractive concentrate from matrix of *A. annua* with AMF inoculation.

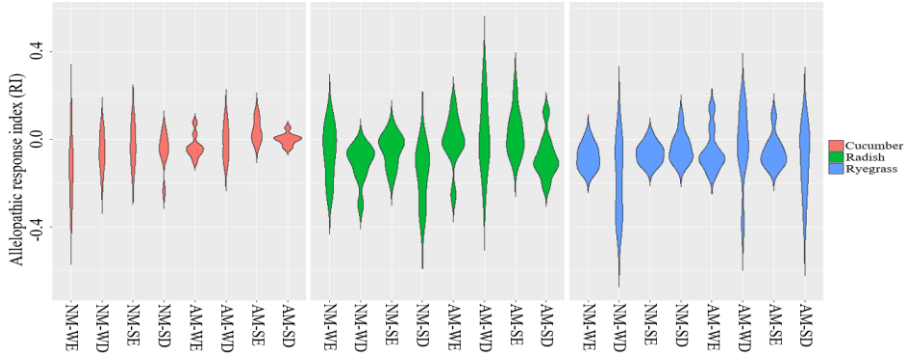

Figure 3. Effects of treatments on Allelopathic response index (RI) of radish, ryegrass and cucumber. NM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* without AMF inoculation; AM-WE: Root exudates obtained by extractive concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* without AMF inoculation; AM-SE: Root exudates obtained by extractive concentrate from matrix of *A. annua* with AMF inoculation; NM-WD: Root exudates obtained by direct concentrate from hydroponic solution of A. *annua* without AMF inoculation; AM-WD: Root exudates obtained by direct concentrate from hydroponic solution of *A. annua* with AMF inoculation; NM-SD: Root exudates obtained by direct concentrate from matrix of *A. annua* without AMF inoculation; AM-SD: Root exudates obtained by direct concentrate from matrix of *A. annua* with AMF inoculation

Heat-map analysis combined with hierarchical cluster analysis (Figure 4) showed that cluster 'a' was more inhibitory to recipient plants, which correlate with AM, while cluster 'b' had weak inhibitory or stimulatory effects on recipient plants, which correlate with NM. Besides, the main factors that affects the cluster 'a' and cluster 'b' classification were shoot-related index (cluster 'c') and root-related index (cluster 'e'), whereas, germination-related index (cluster 'd') had little influence.

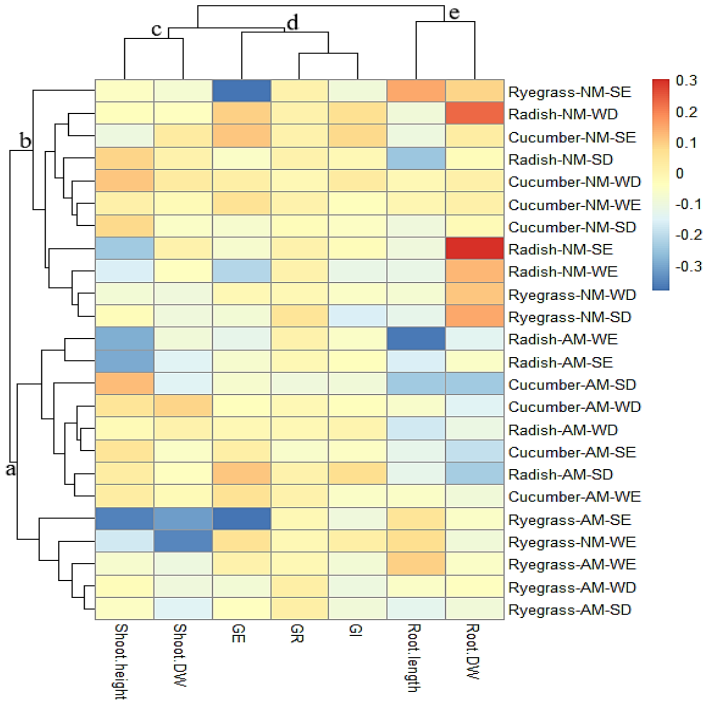

Figure 4. Heat-map analysis combined with hierarchical cluster analysis of the allelopathic response index (RI) for shoot height, shoot DW, germination potential (GE), germination rate (GR), germination index (GI), root length and root DW in response to different treatments

Symbiosis of AMF and plants, stimulates the absorption of nutrients from soil and the secretion of root exudates by host plants (11,14,25). Cluster analysis showed that root exudates of *A. annua* inoculated with AMF significantly inhibited the receptor plants than *A. annua* without AMF inoculation. AMF improved the growth conditions of *A. annua*, increased the content of root exudates and thereby enhanced the allelopathy. AMF also helped the host plants to occupy wider living space to obtain more nutrients. However, it may be harmful to subsequent crops, suggesting that AMF inoculation may be harmful in crop rotation. Furthermore, we found that AM had remarkable inhibitory effects on the shoot-related index (cluster c) and root-related index (cluster e) for recipient plants, which may be due to direct interactions between the root exudates and recipient plant roots. This interaction might have affected the nutrients uptake capacity of recipient plants from soil and thereby affected the shoot growth.

VI. Allelopathic synthesis effects

It was single-facet to use a single index to express the strength of allelopathy and the above shortcomings were overcome in calculating the allelopathic synthesis effects. Figure 5 showed the synthesis effects of *A. annua* root exudates on radish, ryegrass and cucumber. Overall, root exudates were most inhibitory to ryegrass than radish and cucumber. Root exudates obtained by different methods also showed variable inhibitory effects on recipient plants. The inhibitory effects of root exudates obtained by extractive concentrate were stronger than those obtained by direct concentrate. The inhibitory effects of root exudates collected from the matrix were stronger than those collected from the hydroponic solution.

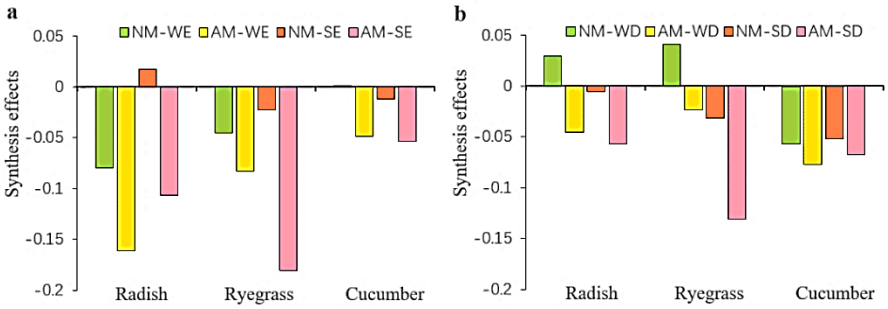

Figure 5. Effects of **a** extractive concentrate and **b** direct concentrate on Allelopathic synthesis effects on radish, ryegrass and cucumber.

Allelochemicals residues after growing of *A. annua* will adversely affect the growth of subsequent crops. The sensitivity of different receptor plants to *A. annua* root exudates was variable. Ryegrass was more sensitive to root exudates than radish and cucumber, hence, its growth was inhibited strongly. We can design herbicides and plant growth regulators according to the sensitivity of different recipient plants root exudates (7). On the other hand, the results of allelopathic bioassay were also related to the collection methods of root exudates. Our study found that root exudates obtained by extractive concentrate were most inhibitory to recipient plants than direct concentrate method, which was consistent with Tang *et al.* (27), indicating that root exudates were easily extracted by ethyl acetate. The inhibitory effects of root exudates collected from the matrix were more inhibitory than these collected from the hydroponic solution, suggesting that root exudates were mainly retained in the matrix. Root exudates obtained by different methods can be used to optimize the allelopathic experiments.

CONCLUSIONS

Allelochemicals residues after planting *Artemisia annua* L. may affect the growth of subsequent crops. The main component of *A. annua* root exudates was dibutyl phthalate (identified by GC-MS). The root exudates were inhibitory to germination rate, germination potential, germination index, shoot height, root length, shoot dry weight and root dry weight of recipient plants. The *A. annua* root exudates may be harmful to subsequent crops, thus it might not be suitable for rotation with radish, cucumber and ryegrass. The root

exudates may be developed as weedicide in future research. The content of root exudates increased in AM than with NM, which enhanced the allelopathy of *A. annua* Inoculation with AMF is beneficial to host plants, but is harmful to subsequent crops. Root exudates from extractive concentrate method were more inhibitory than those from direct concentrate method, which means the root exudates were easily extracted by ethyl acetate. The root exudates collected from the matrix were more inhibitory to recipient plants than collected from hydroponic solution, indicating that more root exudates can be retained in the matrix.

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