https://doi.org/10.26651/allelo.j/2019-47-2-1234 0971-4693/94 Euro 20.00
Allelopathy Journal 47 (2): 233-242 (July, 2019) *International Allelopathy Foundation* 2019
Table: 1, Figs : 3, Photograph : 1

Allelopathic potential of volatile organic compounds released by *Xanthium sibiricum* Patrin ex Widder

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(Received in revised form: May 01, 2019)

ABSTRACT

To evaluate whether *Xanthium sibiricum* releases volatile organic compounds (VOCs) to facilitate its dominance via allelopathy, we investigated the chemical composition and the allelopathic activity of its VOCs, volatile oil and 2 major constituents of the oil. In total 21 compounds (accounting for 96.59% of the total oil) were identified with GC-MS. The most abundant compounds were : limonene (56.89%), β-myrcene (26.23%), cadina-1,4-diene (4.50%) and sabinene (1.83%). These 4 compounds represented 89.45% of the total oil, whereas, other compounds < 1% in the oil. Fresh aerial parts of *X. sibiricum* at 80 g/1.5L in an air-tight container suppressed the root growth of receiver plants *Amaranthus retroflexus* L. and *Poa annua* L. by 49.1% and 69.6%, respectively. The volatile oil exhibited strong inhibitory activity to seedling growth of both species; at 5µg/mL, root elongation of *A. retroflexus* was nearly completely inhibited, while *P. annua* was suppressed by 81.5%. However, the most abundant constituents, i.e. β-myrcene and limonene and their mixture, showed much weaker activity than oil, these inhibited the root growth of *A. retroflexus* by 24.5 %, 59.6 % and 44.8 %, respectively and *P. annua* by 25.1 %, 60.5 % and 44.5 %, respectively. Our results indicated that VOCs produced by *X. sibirium* might contribute to its dominance, and it has the potential to be further explored as a bioherbicide.

Key words: Allelopathy, *Amaranthus retroflexus*, bioherbicide, limonene, β-myrcene, *Poa annua*, VOC, volatile oil, *Xanthium sibiricum.*

INTRODUCTION

The genus *Xanthium* (family: Compositae) comprises of 25 species, originated from South America and now distributed in many parts of the world (30). *Xanthium* species have distinct spiny fruits, which get attached to the grazing sheep and it facilitates this plant's quick spread (3). *Xanthium* plants can adapt to various environmental conditions and if light, moisture and nutrition are sufficient readily form pure stands. Young *Xanthium* plants are poisonous and unpalatable to animals (33). In fact, some *Xanthium* species (*Xanthium italicum, X. spinosum*, etc.), are considered invasive species worldwide, and some of them are even listed on the quarantine pests list in China (27,35).

Allelopathy has been suspected of contributing to the dominance of *Xanthium* plants (26,27). Allelopathy refers to any direct and indirect harmful or beneficial effect of one plant on another through the production and release of chemical compounds into their nearby environment (22). Studies suggest that allelopathy may facilitate the establishment of dominance of number of plants, including some notorious exotic species (6,24,25). The responsible allelochemicals are often categorized as secondary metabolites [phenolics, alkaloids, flavonoids and volatile organic compounds (VOCs) (5,13)]. Allelochemicals isolated and identified from *Xanthium* plants are : xanthatin and its analogues such as 1α,5α-

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epoxyxanthatin, 4-epiisoxanthanol, 4-epixanthanol, as well as other compounds such as loliolide, dehydrovomifoliol, phenolics, and volatile oils (26,35).

Photograph 1. *Xanthium sibiricum* plant and seeds

Xanthium sibiricum is an annual weed distributed throughout China. It has been used in traditional Chinese medicine to treat nasal sinusitis, headaches, appendicitis, chronic bronchitis and rheumatism (15,29). Phytochemical studies revealed the presence of numerous biologically active compounds [xanthanolides, alkaloids, flavonoids, saponins, phytosterols, tannins, phenols, VOCs, etc.] in most *Xanthium* plants (7,29). VOCs can either function directly in their volatile gaseous form, or accumulate in soil to negatively affect the neighbouring plants growth (5,14). Most *Xanthium* plants produce VOCs, but their constituents differ significantly. Volatile oils possess many biological activities, antimicrobial, antioxidant, anti-inflammatory and herbicidal; however, the herbicidal activity of *X. sibiricum* essential oil has never been studied (4,8,13,16). The volatile oils of *Xanthium* plants possess strong phytotoxic activity, indicating the possible involvement of volatile oils as allelopathic agents to suppress neighbouring plants growth (27). This study aimed to evaluate the allelopathic potential of VOCs produced by *X. sibiricum*, the chemical composition and phytotoxic activity of its volatile oil, which could help us to better understand the complicated mechanism of its dominance.

MATERIALS AND METHODS

Materials

Aboveground plant parts of *X. sibiricum* at vegetative stage were collected in July, 2016 near a corn field in Urumqi, Xinjiang Uygur Autonomous Region, China (N43°54′40.1″, E87°17′7.9″). Specimens were identified by Dr. Wenjun Li from Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences. A voucher specimen (XJBI 016225) was deposited with the herbarium of Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences. Fresh plant materials were used directly for the allelopathic activity assay of VOCs and extracting volatile oil. Limonene (analytical standard) was purchased from Sigma-Aldrich Co. (St. Louis, USA), and β-myrcene (analytical standard) were purchased from Aladdin Co. (Shanghai, China).

Potential allelopathic effect of VOCs

Fresh aerial plant parts of *X. sibiricum* were put into plastic containers (13.5 cms × 13.5 cms × 8.5 cms, volume 1.5 L) at 0, 10, 20, 40 and 80 g per 1.5 L container. The allelopathic effects of the VOCs were evaluated by conducting bioassays against dicot plant (*Amaranthus retroflexus*) and a monocot plant (*Poa annua*) sharing the same habitat with *X. sibiricum*. Seeds of two targeted species were surface sterilized with 0.5 % $HgCl_2$ before use. Five mL of distilled water was added to each petri dish (9-cm dia) lined with layer of whatman filter paper, followed by sowing of 10 seeds equidistant from each other. The petri dishes were kept open without lids. Lids of containers were removed for 15 min each day to allow in fresh air for the seedlings. Containers were kept in dark incubator at 25 ℃, and root and shoot lengths were measured 4 days (for *A. retroflexus*) and 7 days (for *P. anuua*) after incubation. The treatments were replicated thrice in phytotoxic bioassays (Total 30 seedlings shoot and root were measured).

Isolation of volatile oil

Fresh plant material of *X. sibiricum* (200 g) was hydrodistillated for 3 h using a Clevenger type apparatus to isolate the volatile oil. The oil was dried over anhydrous sodium sulfate and kept in a sealed vial at 4℃ until use. The extraction procedure was repeated until sufficient oil was obtained for GC/MS analysis and phytotoxic assays.

Gas chromatography-mass spectrometry analysis

The oil was analyzed by GC/MS using a Perkin-Elemer Autosystem XL-Turbemass system with a PE-5MS capillary column (60m×0.25mm; 0.25 μm film thickness) thickness). The carrier gas was helium, with a flow rate of 1 mL/min. The oven temperature was held at 60℃ for 5 min then programmed at rate of 2℃/min to 270℃ and then held at this temperature for 3 min. Mass spectra were taken at 70 ev. Mass range was from m/z 35-350 amu. Injector port temperature: 280℃, detector: 280℃, injected volume: 0.1 μL, split ratio: 1:50. Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction. Identification of the constituents of the volatile oil was made by comparison of their mass spectra and retention indices (RI, calculated by linear interpolation relative to retention times of a standard mixture of C8-C40 *n*-alkanes) with NIST and a user library.

Phytotoxic effect of volatile oil and its major constituents

In bioassay the phytotoxic effects of volatile oil, its major constituents [limonene (56.89%) and β-myrcene (26.23%)] and their mixture in the same ratio as in the oil (56.89: 26.23), were evaluated on test plants: *A. retroflexus* and *P. annua*. Seeds of test species were surface sterilized with 0.5% $HgCl_2$ before use. The volatile oil, limonene, β-myrcene and their mixture were diluted in 0.5% acetone and in distilled water to give 0.1, 0.5, 2.5, 5 µg/mL solutions, with acetone as the initial solvent (previous study showed that acetone at such concentration did not affect the seedling growth of receiver plants; 23). Five ml 0.5% acetone in distilled water (control) or diluted solutions were added to each petri dish (9 cms dia), followed by sowing of 10 test seeds equidistant from each other. Petri dishes were sealed with parafilm to prevent water loss and oil volatilization and placed in an incubator in dark at 25 °C. Root and shoot height were measured 4 days (*A. retroflexus*) and 7 days

(*P. anuua*) after incubation. The treatments were replicated thrice for the phytotoxic bioassays (Total 30 seedlings were measured).

Statistical analysis

The significance of allelopathic/phytotoxic effects of the fresh plant parts, the volatile oil, limonene, β-myrcene, and their mixture on seedling growth of test species was first examined by ANOVA ($p < 0.05$) and then analyzed using Fisher's LSD test at $p <$ 0.05 level. The statistical analyses were performed using SPSS 13 software package.

RESULTS AND DISCUSSION

Potential allelopathic effect of VOCs

VOCs released by fresh *X. sibiricum* plant parts significantly inhibited the seedlings growth of receiver species. In terms of root growth, *P. anuua* was more sensitive (26.4 % suppression in root length) when treated with 20 g fresh *X. sibiricum* plant parts /1.5L container, whereas, at the same rate the inhibition in root length of *A. retroflexus* was only 8.7 %. At 40 g fresh *X. sibiricum* plant parts /1.5L container, root length of *A. retroflexus* and *P. annua* was reduced by 35.3% and 47.7 %, respectively. While at 80 g/1.5 L container fresh plant material resulted in reduction of 49.1% and 69.6%, respectively, in root growth of *A. retroflexus* and *P. annua* (Figure 1). Shoot growth of tested plants was also affected by VOCs in a dose-dependent manner but to a lesser extent (data not shown).

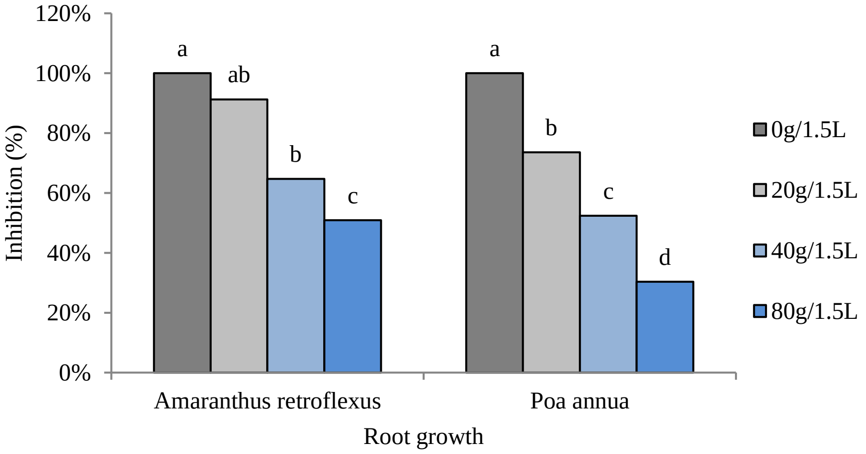

Figure 1. Allelopathic effects of VOCs released by fresh *X. sibiricum* plant materials on seedling growth of *Amaranthus retroflexus* and *Poa annua* (N=30). Means with different letters indicate significant differences at p<0.05 level according to Fisher's LSD test.

Chemical composition of volatile oil

The volatile oil of *X. sibiricum* obtained by hydrodistillation of fresh aerial plant parts had yellow colour with an herbal odour. The yield was 0.15% (v/w, volume/fresh weight). In total 21 compounds were identified, accounting for 96.59% of the total oil, while 3.41% of the oil remained unidentified (Table 1). Monoterpenes and sesquiterpenes were the dominant constituents in the total oil. Among the 21 oil constituents, the most abundant compounds were : limonene (56.89%), β-myrcene (26.23%; Figure 2); another 2 relatively abundant compounds were cadina-1,4-diene (4.50%) and sabinene (1.83%). These 4 compounds represented 89.45% of the total oil, whereas each remaining compounds < 1% in oil.

Table 1. Volatile oil composition of *X. sibiricum*

Number	Compounds	Retention time (min)	Retention Index	Percentage (%)	Molecular formula
1	Camphene	6.94	944	0.10	$C_{10}H_{16}$
2	Sabinene	7.81	969	1.83	$C_{10}H_{16}$
3	β-Myrcene	8.69	995	26.23	$C_{10}H_{16}$
4	Limonene	10.87	1038	56.89	$C_{10}H_{16}$
5	Nonanal	14.39	1104	0.16	$C_9H_{18}O$
6	3,5-Nonadien-7-yn-2-ol	19.07	1178	0.25	$C_9H_{12}O$
7	Bornyl Acetate	25.88	1280	0.11	$C_{12}H_{20}O_2$
8	δ-EIemene	29.35	1322	0.60	$C_{15}H_{24}$
9	β-Elemene	32.85	1385	0.13	$C_{15}H_{24}$
10	Caryophyllene	34.77	1414	0.92	$C_{15}H_{24}$
11	α-Bergamotene	35.71	1429	0.08	$C_{15}H_{24}$
12	α-Caryophyllene	36.99	1449	0.37	$C_{15}H_{24}$
13	α-Neoclovene	38.08	1466	0.13	$C_{15}H_{24}$
14	Germacrene D	38.75	1476	0.99	$C_{15}H_{24}$
15	Cadina-1,4-diene	40.52	1504	4.50	$C_{15}H_{24}$
16	β-Sesquiphellandrene	41.55	1521	0.40	$C_{15}H_{24}$
17	Germacrene B	43.42	1551	0.10	$C_{15}H_{24}$
18	Spathulenol	43.91	1559	0.97	$C_{15}H_{24}O$
19	Santalol	46.16	1596	0.44	$C_{15}H_{24}O$
20	Ledene oxide	48.01	1627	0.87	$C_{15}H_{24}O$
21	Phytol	73.48	2108	0.52	$C_{20}H_{40}O$

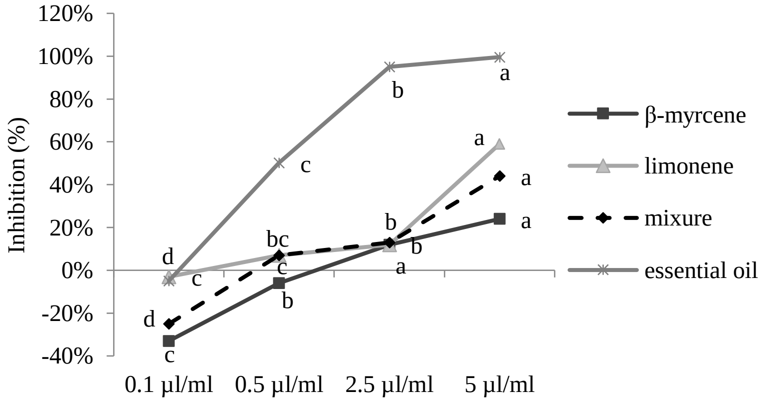

Figure 2. Phytotoxic effects of β-myrcene, limonene and their mixture as well as the volatile oil of *X. sibiricum* on root growth of *Amaranthus retroflexus.* Different letters represent a significant difference at $p < 0.05$ level according to Fisher's LSD test. Each value represents the inhibition rate (%) compared to the control.

Phytotoxic activity of volatile oil and its major constituents

The phytotoxic activity of volatile and its major constituents (limonene and β-myrcene, and their mixture, were determined by comparing their effect on shoot and root length of two receiver plants, *A. retroflexus* and *P. anuua*.

(i). *A. retroflexus:* β-Myrcene and the mixture of limonene and β-myrcene significantly promoted the root growth of *A. retroflexu*s by 33.2 % and 25.7 % at 0.1 µg/mL, respectively. The limonene did not promote activity, of mixture was initiated by the compound β-myrcene. At 2 µg/mL concentration, both limonene and β-myrcene inhibited the root elongation of both receiver plants. While at 5 µg/mL concentration, the limonene, β-myrcene and their mixture suppressed the root length of *A. retroflexu*s by 24.5%, 59.6% and 44.8%, respectively. The inhibitory activity of volatile oil was much stronger than limonene, β-myrcene and their mixture. The volatile oil at 0.5 µg/mL reduced the root growth of *A. retroflextus* by 50.1 % than control. The seed germination was completely inhibited at 2 µg/mL and 5 µg/mL oil (Figure 3).

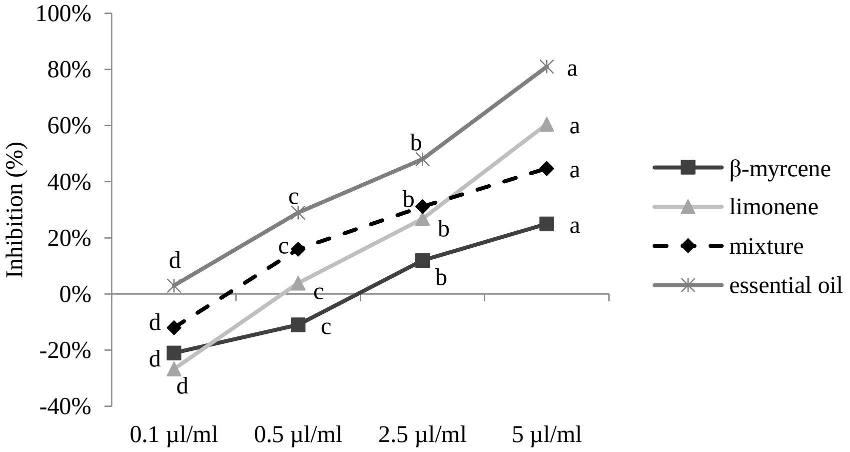

Figure 3. Phytotoxic effects of β-myrcene, limonene and their mixture as well as the volatile oil of *X. sibiricum* on root growth of *Poa annua*. Different letters represent a significant difference at $p < 0.05$ level according to Fisher's LSD test. Each value represents the inhibition rate (%) compared to the control.

(ii). **Poa annua:** Similar to *A. retroflextus*, β-myrcene, limonene and the mixture of these 2 compounds enhanced the root elongation of *P. annua* at the lowest concentration, 0.1µg/mL. While 2.5µg/mL, β-myrcene, limonene and the mixture suppressed root growth by 12.5%, 26.9%, 16.5%, respectively. At 5 µg/mL concentration the root growth was further reduced to 25.1%, 60.5%, and 44.5%. Similarly, the volatile oil at 0.5, 2.5 and 5µg/m reduced the root length by 29.2%, 48.2% and 81.5%.

Constituents of volatiles (β-myrcene, limonene, camphor, eucalyptol, thymol, geraniol, α- and β-pinene, bornyl acetate, citronellal, menthol, borneol and a-terpineol) inhibits the plant growth (20,36). In a study evaluating the phytotoxic activity of 27 monoterpenes at 10^{-3}~10^{-6} M, 10 and 4 monoterpenes significantly decreased the germination of radish and garden cress, whereas 4 and 6 monoterpenes drastically reduced the radical elongation of radish and garden cress, respectively (20). There are successful examples of utilizing oil constituents as commercial herbicides. For example, cinmethylin is derivative of 1,4-cineole which is a natural phytotoxin found in the volatile oils of many plants. Clove oil (common volatile oil constituent), is major component in commercial herbicide Burnout II (Bonide Products Inc., New York; 1,12)

Information is available on the chemical composition of *Xanthium* species, the β-myrcene and limonene are found in most of these oils. Although the relative percentage of β-myrcene varied greatly among the different species, limonene was abundant in oil of many *Xanthium* species [*X. cavanillesii* (43.6%), *X. pennsylvanicum* (65%), *X. pungens* (40.2%), *X. strumarium* (24.7%), *X. italicum* (35.3%) from Corsica and *X. italicum* (51.61%) grows in China (2,10,26,27)]. We found 21 compounds in the volatile oil of *X. sibiricum*, with limonene (56.89%), β-myrcene (26.23%), cadina-1,4-diene (4.50%) and sabinene (1.83%) being the major constituents; however, the chemical composition of *X. sibiricum* growing in other provinces was different from our results. For example, the major ingredients of the essential oil of *X. sibiricum* collected in Guangxi province were : 9 , 12 - Octadecadienoicacid (22.1%), hexadecanoicacid (14.0%), Hunulene (10.55%) and Caryophyllene (10.44%), whereas the major compounds were sabinene (43.07%), linolenic acid (11.64%) and hexadecanoic acid (7.75%) for *X. sibiricum* oil collected in Shandong province, and the major components were (1E,6E,8S) -1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene (28.49%), phytol (12. 253%) and caryophyllene (5.237%) for the same plant growing in Hainan province (19,21,34).

Aqueous extract of *X. sibiricum* is allelopathic to receiver species seedling growth, (*Rassica campestris* and *Raphanus sativus*) (11). Although some biological activities (anti-tumor, anti-microbial, anti-oxidant activities) of essential oil of *X. sibiricum* have been reported, but our study was the first to report its allelopathic effects. We have detected 21 compounds in the essential oil, which makes it difficult to ascribe its biological activities to any certain constituents. The mixture of major constituents of essential oil caused slight inhibition in receiver plants than essential oil, indicating that ingredients other than these compounds in the volatile oil play important role. Further research is needed in this direction. Our results implied that *X. sibiricum* oil has the potential value for use as bioherbicide; however, there are certain issues limiting the direct use of naturally occurring substances as herbicides. For instance, they have weak herbicidal activity, their yields are often not high, and sometimes they are not stable enough to be applied under natural conditions (24). Meanwhile, the fact that *X. sibiricum* can produce volatile compounds and release them into the environment indicated the possible role of allelopathy contributed to the dominance of *X. sibiricum*. However, the mechanism might be quite complicated. Volatile phytotoxins may result in a series of ecological consequences other than their toxicity alone, which may subsequently alter the soil properties. It was reported that phytotoxins added into the soil had important impacts on soil nutrients dynamics [organic matter dynamics and nutrient cycling (18,32)]. The phytotoxins may also alter the soil microbial community structure and function, which presumably could create unfavourable growth conditions for other plants (9,17). Therefore, VOCs released by *X. sibiricum*, possibly along with other allelochemicals, might function directly and indirectly to influence neighbouring plants' growth and foster its dominance.

ACKNOWLEDGEMENTS

This study was financially supported by the Strategic Priority Research Program of Chinese Academy of Sciences (Grant No. XDA2006030201), the National Natural Science Foundation of China (31770586), and the Taishan Scholars Program of Shandong, China (Grant No. ts201712071).

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