J Korean Soc Appl Biol Chem (2015) 58(2):173–183
DOI 10.1007/s13765-015-0020-3

Online ISSN 2234-344X
Print ISSN 1738-2203

ARTICLE

Characterization of secondary metabolite compounds correlated with the seasons in *Artemisia princeps* var. *orientalis* (Pamp.) H. Hara leaves using direct sample injection and gas chromatography–mass spectrometry: contribution to phytotoxicity

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Received: 15 October 2014 / Accepted: 13 January 2015 / Published online: 27 January 2015

Abstract Leaves from a natural population of *Artemisia princeps* var. *orientalis* (Pamp.) H. Hara were collected monthly from April through October and characterized for composition of secondary metabolite compounds and their phytotoxic effects on seed germination and seedling growth of *Achyranthes japonica* and *Lactuca sativa*. The compounds were identified using gas chromatography/mass spectrometry (GC/MS) coupled with a solvent-free solid injector (SFSI). GC/MS analyses of all samples revealed qualitative variability in the composition of secondary metabolites. The greatest number of compounds was identified in July (56) followed by September (30) and April (24), and the lowest number was found in June (2) and August (2). Among 92 compounds, the major compounds were various terpenes (23) (mono-, sesqui, di-, and tri-terpenes) followed by heterocyclic compounds (18) and hydrocarbons (14). The higher the concentration of the secondary metabolites, the lower the seed germination and seedling growth of *A. japonica* and *L. sativa.* Plant samples collected in July and August were most detrimental. Taken together, variability in the secondary metabolites compounds of *A. princeps* var. *orientalis* was verified during different seasons, and the compounds were successfully identified by a combination of SFSI and GC/MS. Notably, the antimicrobial and antioxidative effects were inconsistent throughout the various seasons.

Keywords Seasonal variations · *Artemisia* · Secondary metabolites · Solvent-free injection · GC/MS

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Introduction

Artemisia princeps (Japanese mugwort), a perennial plant native to China, Japan, and the Republic of Korea, has been used as food and medicine by different populations (Toda 2005). Young leaves of *A. princeps* have a characteristic greenish aroma, and the leaves are sometimes blanched and added to soups or rice cake in Japanese cuisine (Hosking 1997) or taken as tea (Keys 1976). The green juice of the leaves is also used in traditional Japanese folk medicine to treat skin injuries (Umano et al. 2000). The leaves are used to treat gastrointestinal disorders in the Republic of Korea

(Kim et al. 1994). *Artemisia* has been the object of numerous chemical and biological studies (Toda 2004; Sarath et al. 2007; Choi and Kim 2013). Toda (2005) reported the antioxidative effects of polyphenols generated from the leaves of *A. princeps* on lipid peroxidation by free radicals in vitro. The anti-inflammatory and analgesic effects of *A. princeps* along with two other edible plants from the Republic of Korea were reported in mice by Park et al. (1994). The presence of a number of volatile compounds including mono and sesquiterpenes has been well documented in *Artemisia* species (Ahmed and Misra 1994; Kim et al. 1994). Some of these compounds are referred to as "allelochemicals", as they are produced by plants, released into the environment, and subsequently affect the growth and development of neighboring plants (Yun and Kil 1992; Einhellig 2002; Chon et al. 2003). Essential oils from the leaves of *A. princeps* were strongly toxic to wheat seed germination when tested alone at a dose of 500 g/L by Liu et al. (2006). Assessing the allelopathic potential of *A. princeps*, Yun and Kil (1992) found severely inhibited elongation, dry weight, and caloric content of seedlings of other plants grown in soil underneath *A. princeps*.

Plants (leaves, flowers, and fruits) are capable of synthesizing thousands of primary and secondary metabolites with diverse biological properties and functions. These compounds play a vital role in the plant life cycle by providing chemical cues to animals, pollinators, and seed disseminators that ensure plant reproductive and evolutionary success (Dudareva and Negre 2005). *Artemisia* is important in the Republic of Korea because of the presence of attractive monoterpenes and essential oils (Kim 1996). The composition of these volatile compounds varies considerably in different phytogeographical regions (Hall and Langenheim 1987), and these compounds are produced in plant tissues at different developmental stages, including flowering, ripening, and maturation (Goff and Klee 2006). Seasonal variations in volatile compounds from *A. princeps* var. *orientalis* (Pamp.) H. Hara have been studied by Kim (1996) and Yun and Choi (2003) using conventional extraction methods such as steam distillation (Umano et al. 2000) or solvent extraction (Yun et al. 2008; Nugroho et al. 2010). However, the major drawbacks of conventional methods are time, consumption of large volumes of solvents, and the cost related to waste disposal. Additionally, target compounds can be oxidized and/or degraded following the lengthy procedure (Abd El-Aty et al. 2008).

Secondary metabolites are easily oxidized by air and/or degraded by heat. Therefore, an extraction technique that isolates the compounds in an intact form without deterioration is needed (Mamede and Pastore 2006). Solvent-free solid injection (SFSI), a vaporization technique, has some advantages over conventional techniques in terms of simplicity and cost effectiveness. SFSI involves directly injecting samples into a gas chromatograph with no prior sample preparation. Therefore, it is an eco-friendly technique because no solvents are consumed (Kim et al. 2006). Additionally, there is a little possibility of losing the volatile compounds/constituents from the tested samples. No detailed characterizations of volatile flavor compounds from *A. princeps* var. *orientalis* (Pamp.) H. Hara using an environmentally friendly technique throughout various seasons have been conducted. Thus, the objective of this present study was to: (a) identify the secondary metabolites produced by *A. princeps* from April through October using gas chromatography/mass spectrometry (GC/MS) coupled with SFSI and (b) evaluate the phytotoxic effects of the compounds on seed germination and seedling growth of both *Achyranthes japonica* and *Lactuca sativa.*

Materials and methods

Plant materials

Fresh leaves of *A. princeps* var. *orientalis* (Pamp.) H. Hara were collected monthly from April through October from the wild near Suncheon National University, Suncheon, Republic of Korea. The collection site was heavily covered with plant material, and samples were taken from five locations within the sites, sealed in plastic bags, and transported to the laboratory pending analysis.

SFSI

A SFSI device manufactured by Han Jin Precision Co. (Gwangju, Republic of Korea) was used to inject the plant materials into the gas chromatograph equipped with a mass spectrometer. Exactly, 1-mg representative sample of *A. princeps* leaves was placed into a soft glass capillary tube (1.2 mm i.d. × 30 mm in length). Both ends of the tube were sealed briefly in a gas flame, and the tubes were placed in the SFSI. The tubes were crushed by lowering the injector plunger to carry the sample analytes onto the GC column in a carrier gas. The SFSI was held at the injection port during the preheating phase until the injector plunger reached the top of the injector septum, which allowed a constant pressure of carrier gas to be maintained during analysis. At no point in the SFSI technique was the extraction conditions optimized. Rather, the experimental variables including injector temperatures and preheating times were predicated based on our experience.

GC/MS analysis

An Agilent model 6890 network GC system equipped with a 5973N mass-selective detector (Agilent Technologies,

Palo Alto, CA, USA) was used to identify the volatile compounds emitted from *A. princeps* leaves. Separation was carried out on an HP-5MS fused silica column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies). The injector and detector temperatures were set at 250 and 300 °C, respectively. Oven temperature was held at 50 °C for 5 min, increased to 300 °C at a rate of 5 °C/min, and then held constant for 10 min. High-purity helium (99.9999 %), at a constant flow rate of 1 mL/min, was used as the carrier gas, and the injection was made using a split mode of 10:1. An electron impact mass spectral analysis was carried out at ionization energy of 70 eV at 250 °C. Detection was performed in the scan mode between 10 and 400 amu at 3.71 scans/s.

Qualitative analysis

The tentative identification of the compounds was based on comparing the obtained mass spectra with those of reference compounds in the Wiley 7N mass spectral database (McLafferty and Stauffer 1989). Percentage peak areas were calculated by dividing the ion counts for a particular peak (detected by MS) by the total ion counts for the entire chromatogram, and this value was expressed as percentage.

Phytotoxic effect of emitted compounds from *Artemisia* leaves

Fifty seeds each of *A. japonica* and *L. sativa* (sensitive species largely employed in the allelopathy studies, Araniti et al. 2013; Amini et al. 2014) were placed on filter paper that was layered on moist, absorbent cotton with sufficient moisture in a 1,800 mL glass chamber to assess the time-dependent effect of secondary metabolites on seed germination and radicle elongation. Different quantities of sliced *Artemisia* leaves (fresh weights of 5, 10, 15, 20, 25, or 30 g) were placed in a glass beaker within the chamber. An identical chamber without sliced leaves was used as a control. The glass chambers were covered with vinyl wrap and placed in a growth chamber maintained at 25 °C during the day (14 h) and 18 °C at night (10 h). Seed germination and radicle elongation were checked according to the method of Kil and Yun (1992).

Results and discussion

SFSI–GC/MS conditions

Based on our experience, a sample preheated for 10 min (preheating time) at 250 °C (injector temperature) was chosen as the favorable condition for SFSI, in which the greatest number of secondary metabolites was emitted (Kim et al. 2006, 2007; Abd El-Aty et al. 2008; Ko et al. 2014). A sampling time of 55 min was selected to provide an acceptable analysis time with the highest yield of compounds for the overall analysis. This is because the sampling profile appeared to depend primarily on the volatility of the compound studied (Hamm et al. 2003).

Identification of secondary metabolites emitted from *Artemisia* leaves

Monthly identification of compounds emitted from the leaves of *A. princeps* was carried out with a GC/MS coupled with SFSI from April through October to demonstrate the variability in composition and concentration of the secondary metabolites. A total of 94 compounds including acids, alcohols, aldehyde, ketones, amides, esters, pyrazines, pyrroles, pyridines, hydrocarbons, and others were identified (Table 1). The presence of heterocyclic compounds suggested the self-protecting capability of the plants. Pyrazine and pyrroles, identified as odorous substances in many plants (Rothschild et al. 1984), act as warning signals for herbivores to the presence of dangerous chemicals, and in other situations as an attractant for edible fruit which simultaneously assists in seed dispersal (Rothschild et al. 2005). Hashidoko et al. (1989) suggested a specific and significant role for sesquiterpenes in plant protection against microbial invasion if tissue is injured.

In the present study, dibutyl phthalate and squalene were found in blank samples, indicating that these compounds were generated from the capillary tube and/or injector line and, thus, were excluded from the total number of compounds. The reported number of compounds detected in the current study was a bit lower than the 132 compounds identified by Umano et al. 2000. This difference might be attributed to the extraction technique; steam distillation under reduced pressure followed by dichloromethane extraction and simultaneous purging and extraction.

Correlation between seasonal variations and secondary metabolites

Representative GC/MS chromatograms for May and July are shown in Fig. 1. The compounds emitted from *Artemisia* were not consistent throughout the seasons of spring, summer, and fall. As shown in Table 1, the greatest number of compounds was emitted in July (56), followed by September (30) and April (24). The number of compounds decreased substantially to 12 in May and declined drastically to six in October and two each in June and August. Clearly, the plant vegetative stages (July and September) were the richest in secondary metabolites compared to the fading stage in October. As secondary metabolite contents varied throughout the year, we expected that the biological

Table 1 Retention times (RT) and chromatographic relative area percentages of the secondary metabolites generated from *Artemisia princeps* identified by gas chromatography/mass spectrometry solvent-free solid injection

Nos.	Compounds	RT	Relative area (%)							
			Blank	April	May	June	July	August	September	October
1	Acetic acid	1.98		1.56	0.47		2.97		0.73	
2	3-Methylbutanal	2.06		0.38			0.65			
3	Pyrazine, methyl-	4.8					0.15			
4	2-Furanmethanol	6.05		0.27			0.17			
5	Pyrazine, 2,5-dimethyl-	7.88					0.03			
6	Pyrazine, 2,6-dimethyl-	7.92					0.06			
7	Butyrolactone	8.03		0.11			0.27			
8	Phenol	10.7			0.13		0.19			
9	Eucalyptol	12.11					0.57			
10	Ethanone, 1-(1*H*-pyrrol-2-yl)-	13.37					0.21			
11	Glycerin	13.66					0.11			
12	2(1*H*)-Pyridinone	15.6		0.03			0.14			
13	3-Pyridinol	15.7					0.34			
14	2-Hydroxypyridine	15.88		0.02	0.07		1.07			
15	4-Pyridinol	16.05		0.05			0.43			
16	L-Borneol	16.4							0.53	
17	4(1*H*)-Pyridinone	16.42					0.32			
18	2(1*H*)-Pyridinone, 3-methyl	17.14			0.05					
19	3-Pyridinol, 6-methyl	17.5					0.26			
20	1,2-Benzenediol (catechol)	18.42			0.15		0.77			
21	3-Cyclobutene-1,2-dione, 3,4-dimethyl-	19.86					0.02			
22	2-Methyl-5,6,7,8-tetrahydroquinoxaline	20.31					0.02			
23	1*H*-indole	20.4					0.08			
24	1,4-Benzenediol (hydroquinone)	20.54		0.07	2.56		0.16			0.48
25	Hexa-2,4-diyn-1,6-diol	21.4				0.06				
26	1,3-Benzenediol (resorcinol)	21.8		0.02						
27	Phenol, 2,6-dimethoxy-	21.93					0.19			
28	*Alpha*-copaene	22.53					0.09			
29	*Beta*-elemene	22.96		0.05			0.12			
30	*Beta*-caryophyllene	23.71		0.26	0.68		1.65		0.69	
31	Germacrene-D	23.91					0.2			
32	Nonanoic acid	23.91								0.22
33	2*H*-1-benzopyran-2-one	24.07							0.86	
34	*Alpha*-bisabolene, *cis*-	24.1				0.76				
35	*Beta*-selinene	24.22		0.25						
36	*Alpha*-caryophyllene (humulene)	24.55					1.14			
37	*Alpha*-amorphene	25.11					0.19			
38	*Alpha*-muurolene	25.2					0.22			
39	*Beta*-cadinene	25.51			0.42					
40	*Alpha*-farnesene, E,E-	25.82					0.24			
41	*Delta*-cadinene	26.26		0.13			0.49			
42	*Alpha*-calacorene	26.75					0.29			
43	*Alpha*-cadinol	29.32					0.58			
44	Pyrrolo[1,2-a]piperazine-3,6-dione	30.86		0.5						
45	2-(Dimethylhydrazono)butanal	31.67							0.02	
46	1,4-Diaza-2,5-dioxobicyclo[4.3.0]nonane	31.82					0.6			

Table 1 continued

Nos.	Compounds	RT	Relative area (%)							
			Blank	April	May	June	July	August	September	October
47	Pyrrolo[1,2-a]piperazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	31.87					0.76			
48	*m*-Chloropropylbenzene	32.8			1.02					
49	2-Hexadecene, 3,7,11,15-tetramethyl-	32.98							0.1	
50	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	33.71							0.34	0.61
51	Neophytadiene	33.11		0.65			0.85		0.48	
52	(E)-6,6-dimethylcyclooct-4-en-1-one	34.42					0.14			
53	Hexadecanoic acid, methyl ester	34.85					0.07			
54	Dibutyl phthalate	35.65	10.43	3.7	1.07		0.58		0.96	1.88
55	Hexadecanoic acid	35.64							0.58	
56	Hexadecanoic acid, ethyl ester	36.15					0.07			
57	9,12,15-Octadecatrienoic acid, methyl ester	37.32		0.14					0.03	
58	1,11-Dodecadiene	38.03					0.08			
59	Ethyl linoleolate	38.15					0.12			
60	Phytol	38.37		0.67			0.48		0.16	
61	2-Hydroxyhexadecanoic acid	38.97							0.12	
62	9-Octadecenamide, (z)-	39.4							0.13	
63	Hexadecanamide	39.58					0.25			
64	1,2-Benzenedicarboxylic acid, butyl-2-methylpropyl ester	40.07								0.56
65	2-Benzyl-3,6-dioxo-5-isopropylpiperazine	40.52		0.29			0.61			
66	2,7-Dioxaspiro[4.4]nonan-3-one	41		0.12						
67	3-Benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	42.28					0.29			
68	Hexanedioic acid, dioctyl ester	42.38		0.13						
69	9-Octadecenamide (z)	42.84							0.07	
70	1-Hexadecene	44.11							0.02	
71	1-Heptadecene	44.37							0.04	
72	1,2-Benzenedicarboxylic acid, diisooctyl ester	45.56					0.19			0.44
73	1,2-Benzenedicarboxylic acid, 3-nitro-	44.75		5.17	1.75				0.66	
74	Farnesol B	46.31							0.11	
75	10-Demethylsqualene	47.05							6.32	
76	1-Dotriacontanol	47.65					0.15			
77	Pyrrolidine, 1-(1-oxo-9,12,15-octadecatrienyl)-	48.73					0.1			
78	*Trans*-geraniol	49.15							0.01	
79	Squalene	49.56	13.55						0.81	
80	Nonacosane	50.48			1.07			0.09		
81	Pentadecane	51.81					0.12			
82	Heneicosane	52.4		0.87						
83	5-Butyldocosane	52.76						0.86		
84	Heptacosane, 1-chloro-	52.79							1.15	
85	Eicosane	53.4					5.4		8.3	
86	Vitamin E	54.18		0.27	0.69		0.23		0.21	
87	Methyl commate E	55.6							1.48	
88	3-Keto-urs-12-ene	56.4							0.41	
89	Stigmasterol	56.7							1	
90	Hexatriacontane	57.5		0.66			0.32		0.63	0.36
91	*Beta*-amyrin acetate	57.58							2.21	
92	*Gamma*-sitosterol	58.06					0.28		1.87	

Table 1 continued

Nos.	Compounds	RT	Relative area (%)							
			Blank	April	May	June	July	August	September	October
93	5(1*H*)-azulenone	59.43					0.15			
94	*Alpha*-amyrin (viminalol)	60.1					0.49		1.08	

effects would also vary (Roussis et al. 2000; Angelopoulou et al. 2002). In this context, Yun et al. (2008) reported that *Artemisia* collected in spring are useful as food or food additives and those collected in summer are useful for the pharmaceutical industry. We agreed with their findings regarding the summer season; however, we differed with respect to the spring season, which is discussed below.

Variation in antimicrobial and antioxidant activities throughout the seasons

Yun et al. (2008) found that the antimicrobial activity of *Artemisia* was weaker in April and October and more pronounced in July, August, and September. In contrast, they found that extracts of plants collected in May and June were most effective for DPPH radical scavenging activity and those from plants collected in August, September, and October showed the weakest activity. Those authors evaluated the entire extract without any characterization. The major compounds in April (>1 %) were 1,2-benzenedicarboxylic acid and 3-nitro (5.17 %), a secondary metabolite with good antimicrobial activity against *Staphylococcus aureus*, *S. epidermis*, *Bacillus subtilis*, *Candida albicans*, and *Aspergillus niger* (Kavitha et al. 2010) and 3-methylbutanal, which is an aldehyde derived from leucine degradation, responsible for a plant flavor similar to cheese. We suggest that plant samples collected in early spring season (April) may also have a pharmacological effect (antimicrobial effect) in addition to being a food source.

1,2-Benzenediol (2.56 %), a compound that contributes to the antioxidant properties of plant/medicinal herbs (Manorenjitha et al. 2013); 1,2-benzenedicarboxylic acid, 3-nitro (1.75 %), a compound with antimicrobial activity (Kavitha et al. 2010), and nonacosane (1.07 %), a compound with antibacterial effects (Shobha and Agrawal 2007), were the major compounds emitted in May. Additionally, some minor compounds, including vitamin E (0.69 %), were detected which has an antioxidative function (Venkata Raman et al. 2012). Several biological activities have been attributed to *beta*-caryophyllene (0.68 %), including anti-inflammatory, antimicrobial, antioxidant, anticarcinogenic, and local anesthetic activities (Legault and Pichette 2007). Taken together, the sum of the major and minor compounds contributing to the antioxidant effects of *Artemisia* in May was higher than those contributing to antimicrobial action.

No major compounds were detected in June, August, or October that could contribute either to antimicrobial or antioxidant effects.

Most of the major compounds detected in July, including eicosane (5.4 %), *beta*-caryophyllene (1.65 %), and *alpha*-caryophyllene (1.14 %) have antimicrobial effects (Legault and Pichette 2007; Keawsa-ard and Kongtaweelert 2012; Venkata Raman et al. 2012). Notably, the greatest number of compounds was generated in July and it may be that all minor compounds had cumulative and synergistic effects.

We identified some compounds in September that could contribute either to antimicrobial effects such as eicosane (8.3 %), *gamma*-sitosterol (1.87 %), and methyl commate (1.48 %; Venkata Raman et al. 2012) or antioxidant effects such as *beta*-amyrin acetate (2.21 %; Fabiyi et al. 2012) and stigmasterol (1 %; Venkata Raman et al. 2012). 10-Demethylsqualene was found at a high level (6.32 %); however, we could not identify its biological activity in the databases. Anti-inflammatory, antidiabetic, and anticancer effects have been reported for *alpha*-amyrin (1.08 %; Venkata Raman et al. 2012). Taken together, the sum of the compounds responsible for antimicrobial activities exceeded those with antioxidant effects, indicating strong antimicrobial and weak antioxidant effects.

Phytotoxic effects

Many chemical components are allelochemicals, which influence either plant–plant or plant–microbial interactions (Eom et al. 2006). Barney et al. (2005) suggested a potential role for the presence of bioactive terpenoids in mugwort (*Artemisia vulgaris*) establishment and proliferation in introduced habitats. On the other hand, Kegode et al. (2012) attributed the reduction in *Solanum melanocerasum* plant height and fresh weight to the allelopathic potential effect of *Artemisia biennis.* Furthermore, Araniti et al. (2013) found that aqueous and methanolic extract of *Artemisia arborescens* strongly inhibited both germination and root growth of lettuce (*L. sativa* L.). In this study, we tested the effects of emitted compounds from *A. princeps* var. *orientalis* on *A. japonica* and *L.*

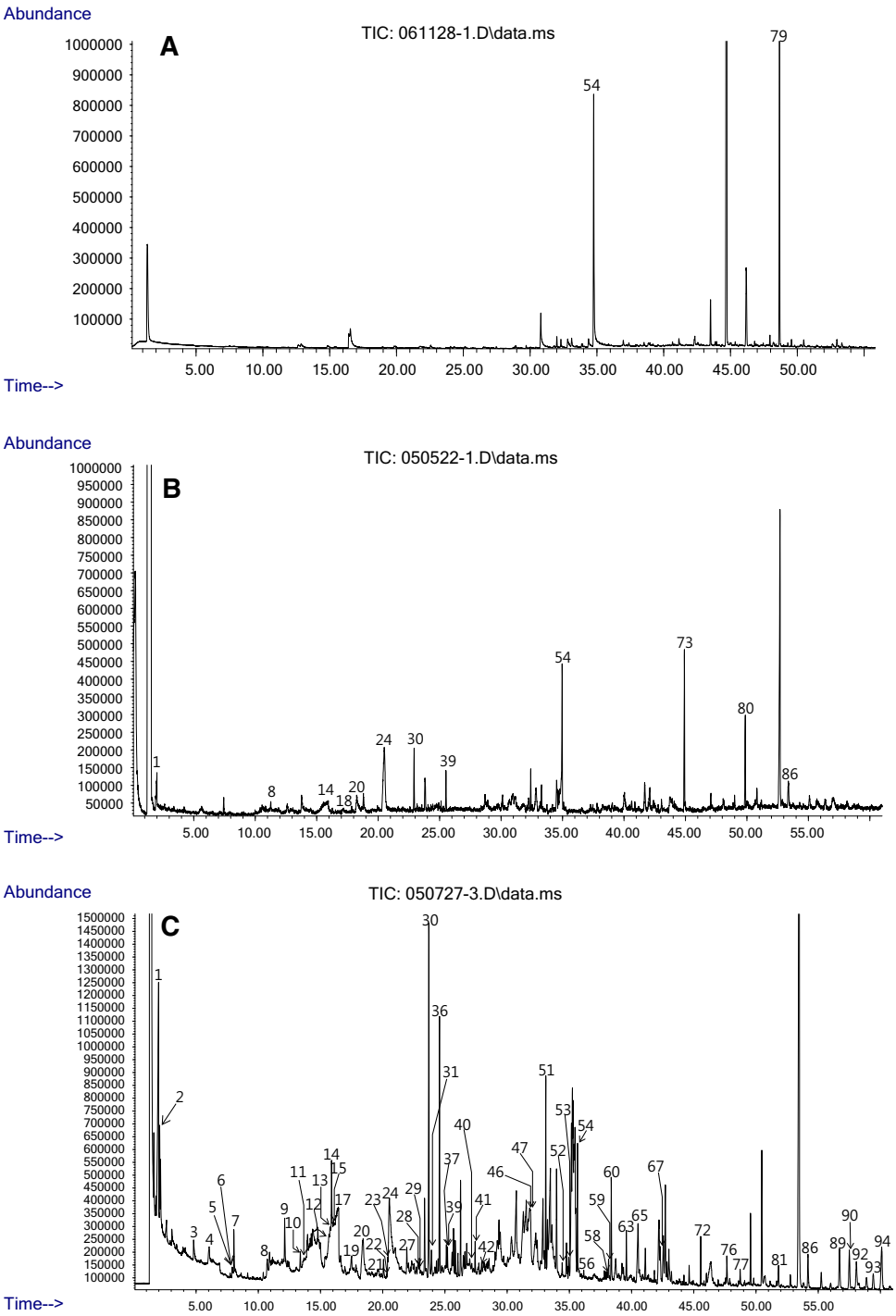


Fig. 1 Typical gas chromatography/mass spectrometry chromatograms of secondary metabolites detected by solvent-free solid injection from *Artemisia princeps* (**a**) blank, during May (**b**) and July (**c**)

sativa. The effects of the secondary metabolites differed depending on the growing season. Neither the amount nor season had a substantial effect on relative germination ratio of *L. sativa*. In contrast, a concentration-dependent inhibitory effect was observed in *A. japonica* when the amount of *Artemisia* was increased, and a severe effect was detected in the summer (particularly August). This result indicates that *L. sativa*, as a crop, is more resistant than *A. japonica*, a representative weed, in response to *Artemisia* secondary metabolites derived from different times of the year (Fig. 2). It is not clear why the effect was most detrimental in August, as only two compounds were generated from *Artemisia* at that time. Could it possible that the emitted compounds are

responsible for the effect? This is the subject of a future study. Another explanation is that the plant collected in summer might be rich in essential oil, which reported to

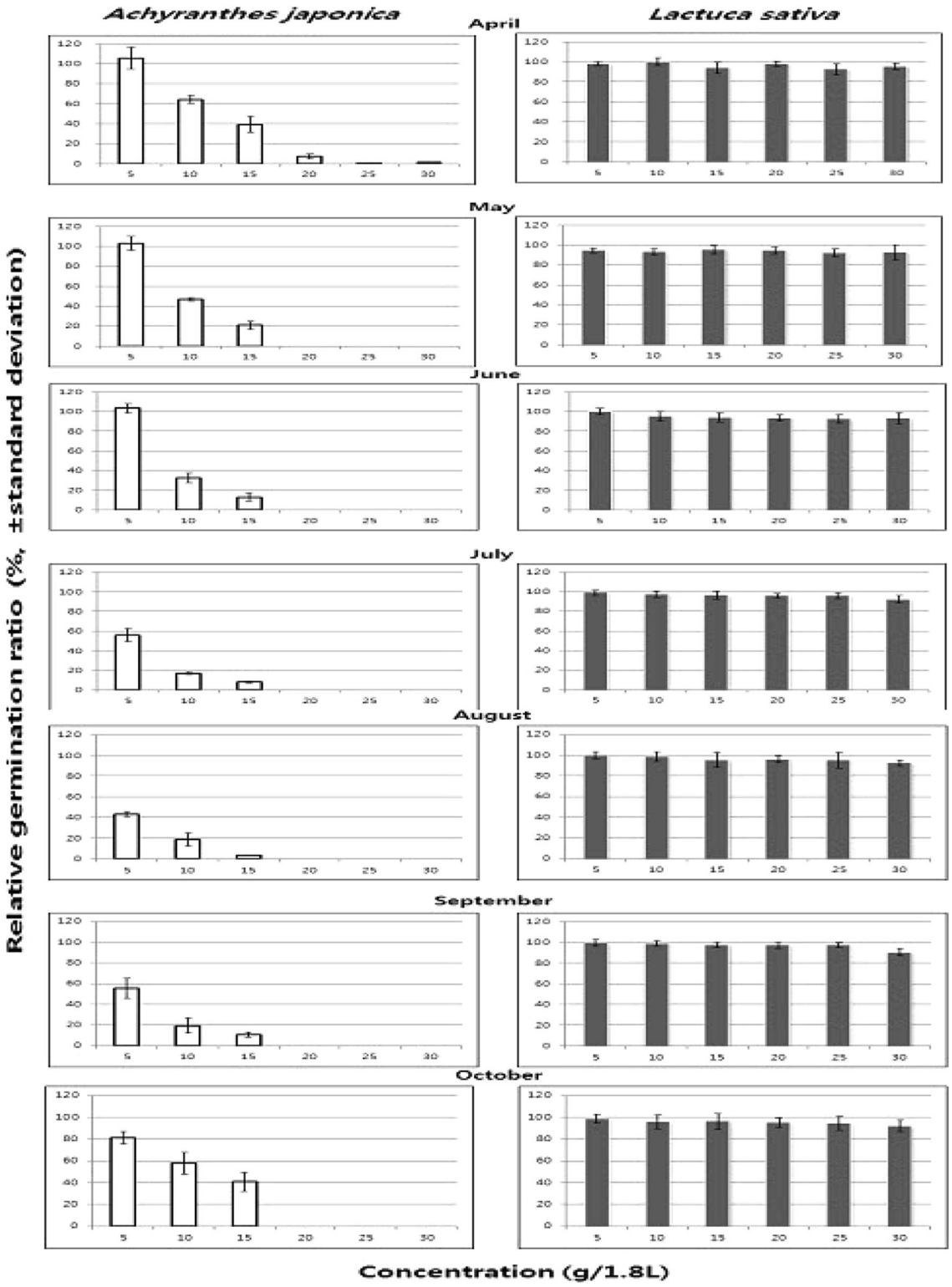
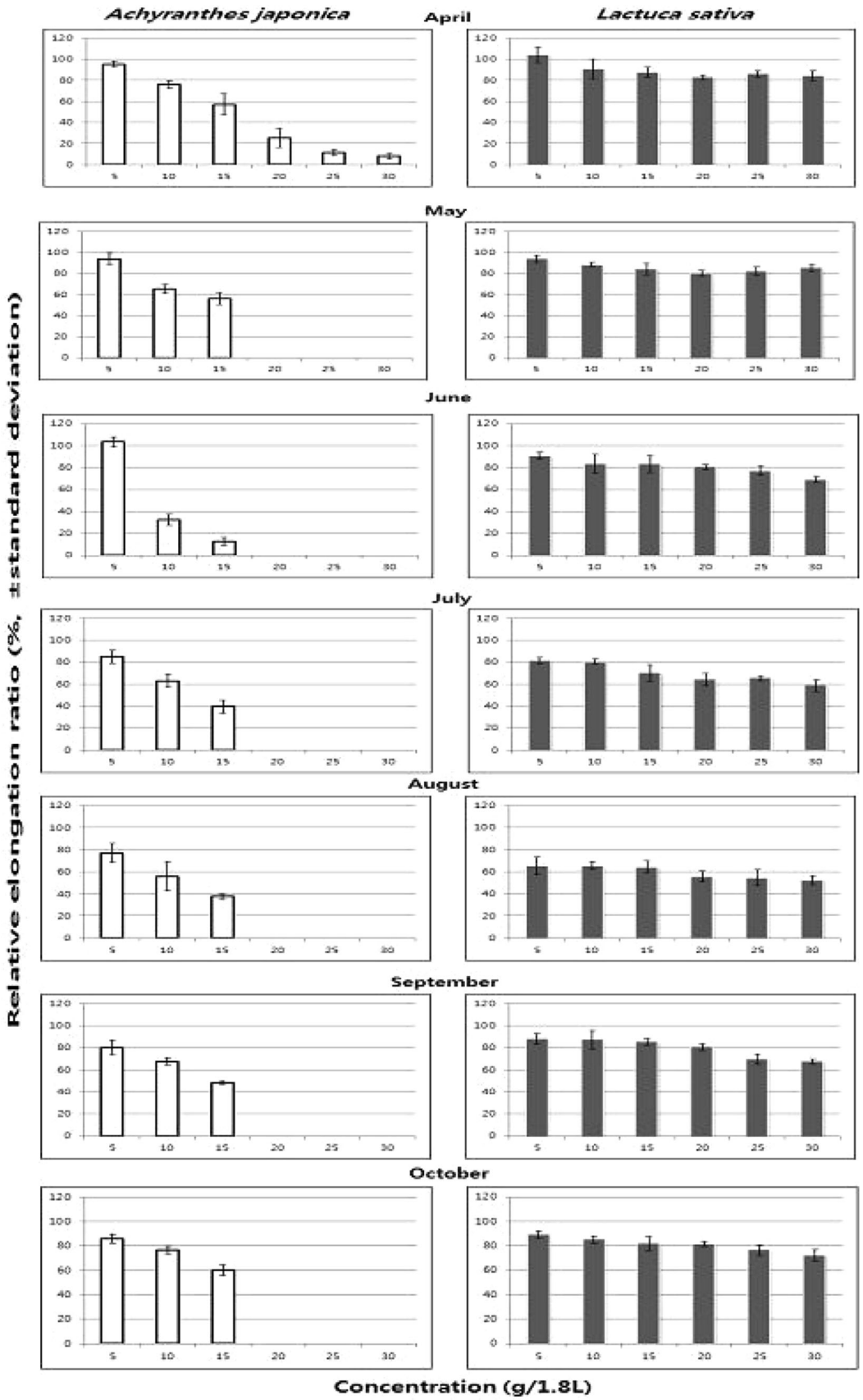


Fig. 3 Relative elongation ratio (±standard deviation) of *Achyranthes japonica* and *Lactuca sativa* sown in a glass chamber, as affected by different concentrations of secondary metabolites emitted monthly from *Artemisia princeps* var. *orientalis*

Fig. 2 Relative germination ratio (±standard deviation) of *Achyranthes japonica* and *Lactuca sativa* sown in a glass chamber, as affected by different concentrations of secondary metabolites emitted monthly from *Artemisia princeps* var. *orientalis*


Achyranthes japonica
Lactuca sativa
April
May
June
July
August
September
October
Relative elongation ratio (%, ±standard deviation)
Concentration (g/1.8L)

have potential phytotoxicity on seed germination and root growth of other plants (Singh et al. 2008; Araniti et al. 2013).

The *Artemisia* concentration had no effect on the relative elongation ratio of *L. sativa*, whereas the inhibitory effect on *A. japonica* was concentration dependent. Notably, the summer season samples affected both plants (Fig. 3). The number of secondary metabolites that were emitted during July and September may be the main reason. It is possible that identified or unidentified components may influence the overall phytotoxic potential of mugwort secondary metabolites emitted over time in an additive/synergistic manner as suggested by Barney et al. (2005).

Seasonal variations in the composition of compounds emitted from *A. princeps* var. *orientalis* (Pamp.) H. Hara and their phytotoxic effects were examined without extraction. The plant materials were injected directly into the GC/MS through a SFSI. As a result, significant seasonal variations in the composition of secondary metabolites were observed. The *Artemisia* plant material showed significant phytotoxic effects on seed germination and seedling growth of *A. japonica* and *L. sativa*.

Acknowledgments This study was supported by the MSIP (Ministry of Science, ICT and Future Planning).

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