Characterization of seed germination stimulants for root parasitic plants produced by cucumber and maize

2014.9

Biological Production Science

United Graduate School of Agricultural Science

Tokyo University of Agriculture and Technology

Pichit Khetkam

Table of Contents

List of Abbreviations

AM fungi	Arbuscular mycorrhizal fungi
COSY	Correlated spectroscopy
DEPT	Distorsionless Enhancement by Polarization Transfer
EtOAc	Ethyl acetate
GC–MS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
HMQC	Heteronuclear multiple quantum correlation
HMBC	Hereronuclear multiple bond correlation
LC–MS/MS	Liquid chromatography tandem mass spectrometry
MeCN	Acetonitrile
MRM	Multiple reaction monitoring
NOESY	Nuclear overhauser enhancement and exchange spectroscopy
NaClO	Sodium hypochlorite
ODS	Octadecylsilyl
t_R	Retention time

Chapter 1. Introduction

1-1. Root Parasitic Plants

Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* spp. and *Phelipanche* spp.) are the two most devastating root parasites, causing severe damages to agricultural production in the world (Parker and Riches, 1993). In sub-Saharan Africa, the Middle East and South Asia, the most damaging root parasites are witchweeds (*Striga* spp.) that parasitize economically important cereal crops such as sorghum, maize, millet, and rice. *Striga* spp. are hemi-parasites that have functional chloroplasts to retain some photosynthetic capacity, but they cannot survive without connecting to host roots and thus they are obligate parasites. In southern Europe, Central Asia and the Mediterranean region, broomrapes (*Orobanche* and *Phelipanche* spp.) are the most damaging root parasites. They are chlorophyll-lacking holoparasites which parasitize a number of important dicotyledonous crops, including tomato, tobacco, carrot, clover, faba bean, cucumber, sunflower and legumes as shown in Fig. 1-1. Root parasites spend most of their life cycle underground and develop flowering stem aboveground. The life cycles of *Striga*, *Orobanche*, and *Phelipanche* spp. are broadly similar, which are closely associated with those of their hosts (Bouwmeester et al., 2003; Xie et al., 2010) as shown in Fig. 1-2.

Fig. 1-1. *Striga hermonthica* infested in maize fields (left) (http://www.push-pull.net/striga.shtml) and *Orobanche cumana* infested in sunflower fields (right) (http://www.agroatlas.ru/en/content/weeds/Orobanche_cumana/).

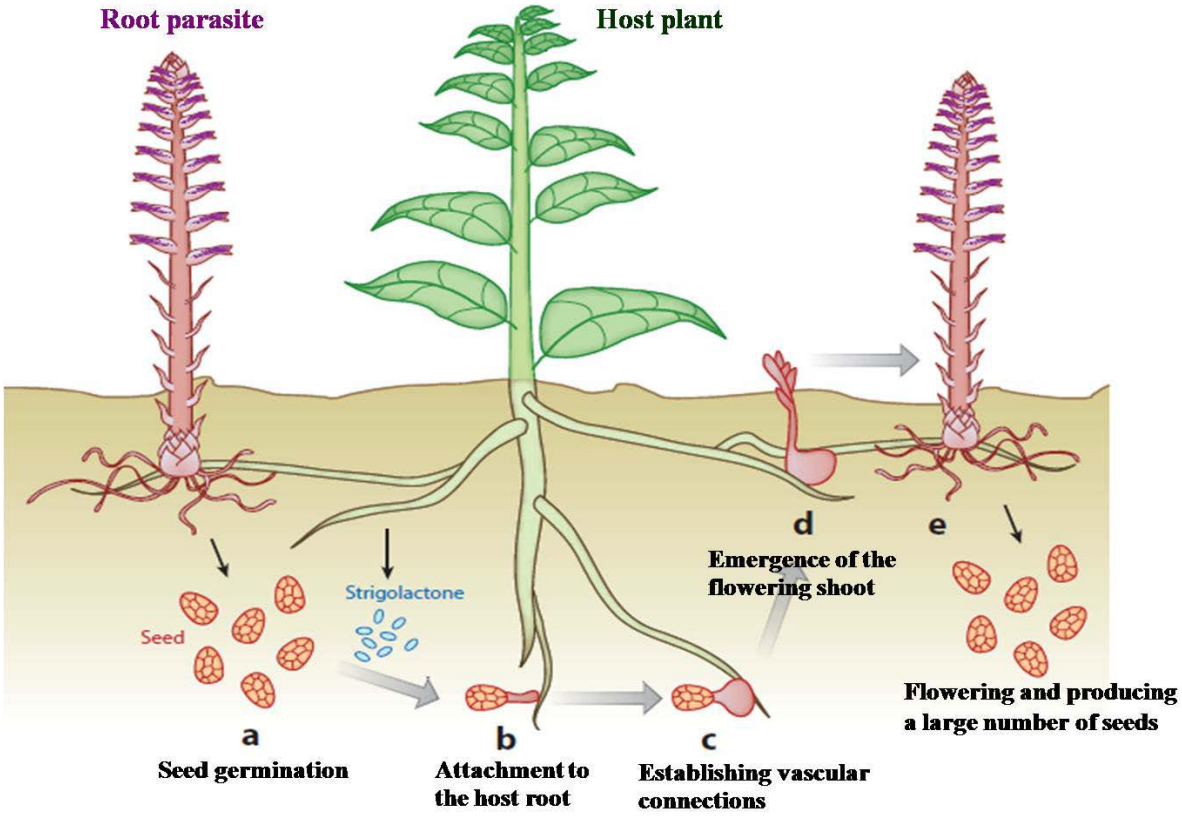


Fig. 1-2. Life cycle of root parasitic plants (Xie et al., 2010).

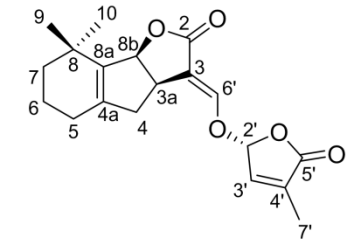
In the rhizosphere, the seeds of root parasitic plants will germinate after a conditioning period under moist environment and suitable temperatures for several days, and only when they are exposed to host-derived signals called germination stimulants produced by and released from host and non-host roots (Joel et al., 1995, 2000, 2007). The radicle of parasite must grow toward a host root, which is possibly directed by the concentration gradient of germination stimulants (Dube and Oliver, 2001). The parasite seedling will develop a haustorium, which is a specialized organ that can attach to the host root and penetrates the epidermis and cortex tissues of the root. The haustorium of root parasites establishes vascular connection with host plant where the parasites can withdraw water and nutrients from the host. In *Striga* spp., the parasite tubercles grow underground for several weeks and for several months in *Orobanche* spp., and further emerge aboveground flowering shoot before producing the flowers and a large number of seeds. Seeds of root parasites are extremely small (*ca*. 200–400 μm), which composed of a relatively small number of cells (Joel et al., 1995). *Striga* and *Orobanche* spp. can produce up to half a million seeds per single plant, which can remain viable in the soil for decades. Such a unique host-dependent life cycle and the production of the numerous long-living seeds in soil make them difficult to control.

1-2. Strigolactones as Germination Stimulants for Root Parasitic Plants and Their Other Biological Functions

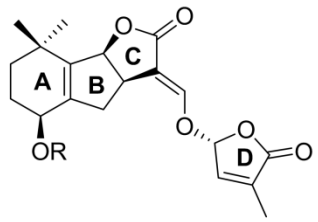
Various germination stimulants for root parasitic plants have been identified in the root exudates of host and non-host plants (Xie et al., 2010). Among them, strigolactones (SLs) are the most potent stimulants, which can induce the seed germination of root parasites at ≤ 10 pM (Kim et al., 2010) as shown in Fig. 1-3. SLs function not only as germination stimulants for root parasitic plants but also as host recognition signals for symbiotic arbuscular mycorrhizal (AM) fungi in the rhizosphere (Akiyama et al., 2005; Bouwmeester et al., 2007; Xie et al., 2010). *In planta*, SLs act as a novel class of plant hormones regulating shoot (Gomez-Roldan et al., 2008; Umehara et al., 2008) and root architecture (Koltai, 2011; Ruyter-Spira et al., 2011), photomorphogenesis (Tsuchiya et al., 2010), secondary growth (Agusti et al., 2011), leaf senescence (Snowden et al., 2005), and induce seed germination of crops and weeds (Pepperman et al., 1988). In addition to these functions in the rhizosphere and in plants, SLs have recently been shown to exhibit inhibitory effects toward growth of breast cancer cell lines (Pollock et al., 2012).

To date, more than 20 SLs have been isolated and characterized from root exudates of various plant species. All natural SLs contain a tricyclic lactone ring system (ABC part) that connects via an enol ether linkage to a butenolide moiety (D ring) (Xie et al. 2010). These SLs have different substituents on the A/B ring but the same C-D moiety and posses an *R*-configuration at C-2′, which have been

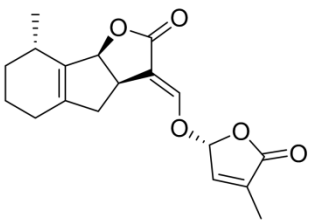
described to be important structural features for inducing high germination of root parasitic plant seeds (Zwanenburg et al., 2009). The natural SLs can be divided into two types based on the stereochemistry or orientation of the C ring. Strigol-type SLs have a β-oriented C ring including strigol (**2**), sorgolactone (**4**), sorgomol (**5**) and strigone (**7**) which are derived from 5-deoxystrigol (**1**). The other is orobanchol-type SLs carrying an α-oriented C ring, including orobanchol (**11**), fabacol (**13**), 7-oxoorobanchol (**15**) and solanacol (**17**), which are derived from 4-deoxyorobanchol (*ent*-2′-*epi*-5-deoxystrigol, **10**) as shown in Fig. 1-3. Although cotton and rice plants produce only strigol-type and orobanchol-type SLs, respectively, some plant species such as tobacco and Chinese milk vetch have been shown to produce both types of SLs (Yoneyama et al., 2013).



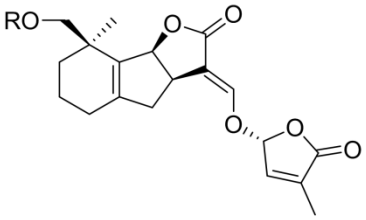
5-deoxystrigol (**1**)



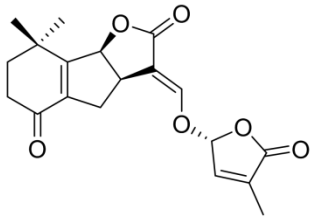
R = H, strigol (**2**)
R = Ac, strigyl acetate (**3**)



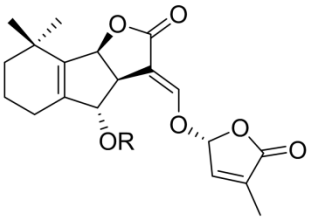
sorgolactone (**4**)



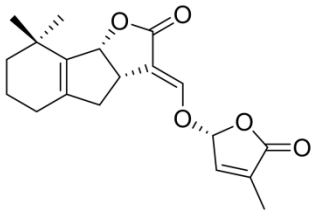
R = H, sorgomol (**5**)
R = Ac, sorgomyl acetate (**6**)



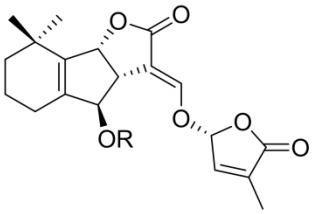
strigone (**7**)



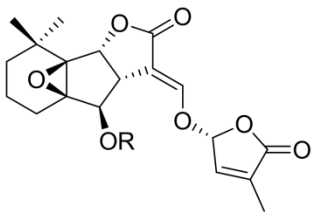
R = H, *ent*-2'-*epi*-orobanchol (**8**)
R = Ac, *ent*-2'-*epi*-orobanchyl acetate (**9**)



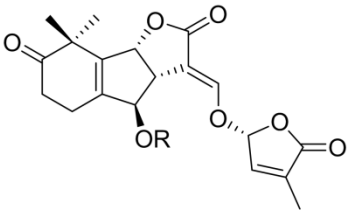
4-deoxyorobanchol (**10**)



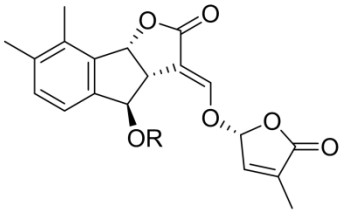
R = H, orobanchol (**11**)
R = Ac, orobanchyl acetate (**12**)



R = H, fabacol (**13**)
R = Ac, fabacyl acetate (**14**)



R = H, 7-oxoorobanchol (**15**)
R = Ac, 7-oxoorobanchyl acetate (**16**)



R = H, solanacol (**17**)
R = Ac, solanacyl acetate (**18**)

Fig. 1-3. Chemical structures of natural SLs produced by plants.

1-3. Biosynthesis of Strigolactones

SLs had been characterized as sesquiterpene lactones until Matusova et al. (2005) demonstrated that SLs would be derived from carotenoid pathway based on the studies using carotenoid biosynthesis inhibitors, fluridone and amitrole, and carotenoid biosynthetic mutants. Recently, Alder et al. (2012) have shown that SLs would be derived from a biosynthetic intermediate called carlactone (**23**), which is formed from β-carotene (**19**) through a pathway catalyzed by three enzymes, Dwarf27 (D27), Carotenoid Cleavage Dioxygenase 7 (CCD7) and CCD8. As shown in Fig. 1-4, the biosynthetic pathway has been proposed to start from the isomerization of the C9–C10 double bond in all *trans*-β-carotene (**19**) to 9-*cis*-β-carotene (**20**) by D27 isomerase. The product, 9-*cis*-β-carotene (**20**) is cleaved at the C-9′–C-10′ double bond by the stereospecific enzyme CCD7 to afford 9-*cis*-β-apo-10′-carotenal (**22**) and β-ionone (**21**), and 9-*cis*-β-apo-10′-carotenal (**22**) is then converted to carlactone (**23**) by CCD8. Formation of the B ring after oxidation of C19 methyl group and further ring closing reaction of carlactone would be catalyzed by More Axillary Growth 1 (MAX1) to afford 5-deoxystrigol (**1**) and its isomer, 4-deoxyorobanchol (**10**), which are common precursors for the other SLs (Alder et al., 2012). The biosynthetic conversion of carlactone (**23**) to SLs was recently proven by using ^{13}C-labeled carlactone (**23**) in rice plants. In addition, endogenous carlactone was detected in both rice and *Arabidopsis* by liquid chromatography-tandem mass spectroscopy (LC–MS/MS) (Seto et al., 2014).

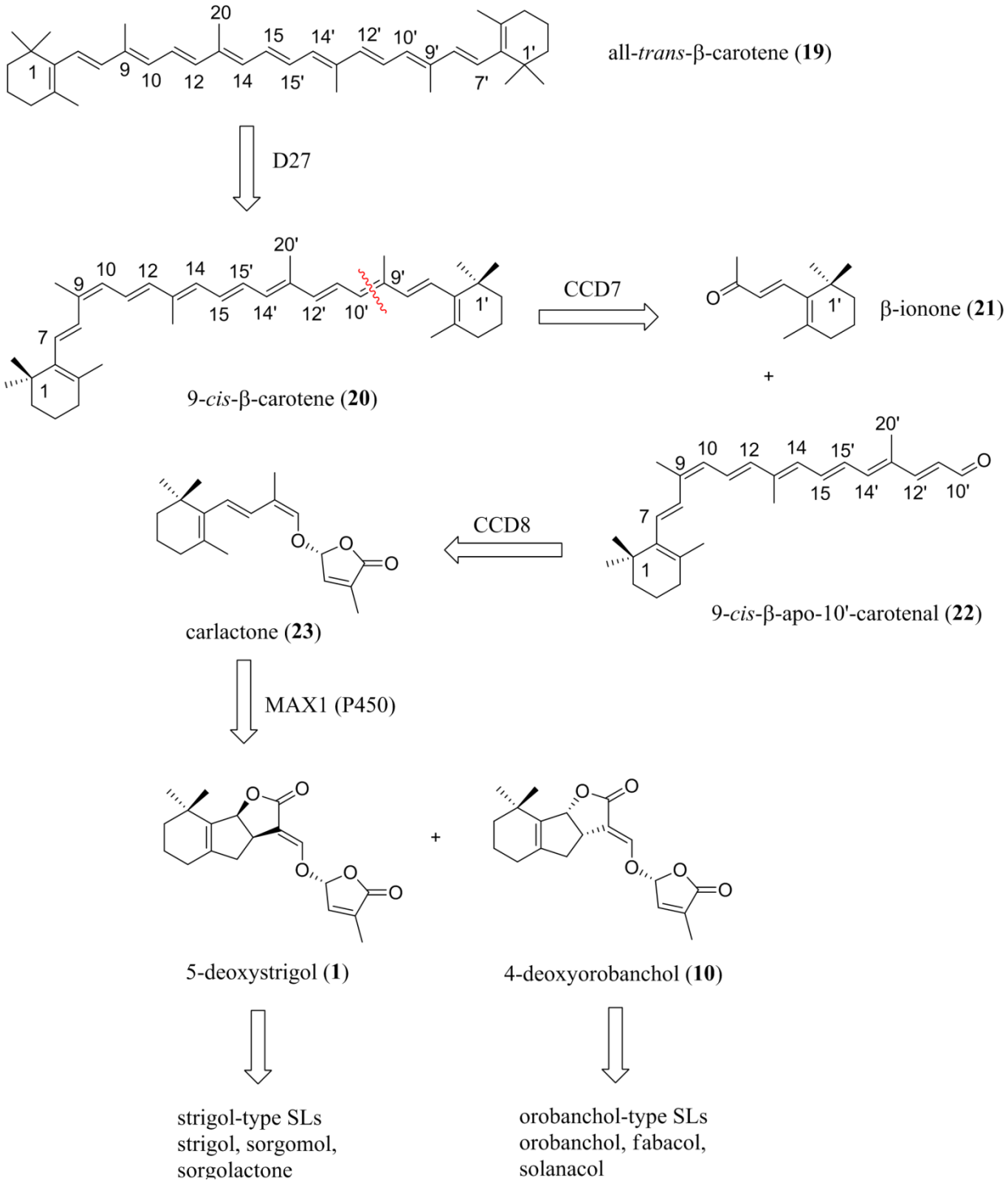


Fig. 1-4. Putative biosynthetic pathway for natural SLs.

Chapter 1 contains general introduction to root parasitic plants and germination stimulants, in particular, strigolactones. In Chapter 2, the isolation and structural determination of novel germination stimulants in cucumber root exudates are described. In Chapter 3, the isolation and characterization of germination stimulants produced by maize are explained. Finally, Chapter 4 includes a summary and discussions.

Chapter2. Isolation and Structural Determination of Novel Germination Stimulants Produced by Cucumber

7-Oxoorobanchol (**15**) and its acetate (**16**) were isolated as germination stimulants for root parasites from flax (*Linum usitatissimum*) (Xie et al., 2009). In addition to these SLs, two novel SLs were detected by LC–MS/MS. Based on their LC–MS spectra and retention times on reversed phase (RP)-HPLC, these SLs were suggested to be hydroxyorobanchyl acetate isomers. Unfortunately, their structures could not be determined due to the scarcity of purified compounds.

In this chapter, isolation and structural determination of novel germination stimulants including two hydroxyorobanchyl acetate isomers, 7α-hydroxyorobanchyl acetate (**26**) and 7β-hydroxyorobanchyl acetate (**27**), from cucumber (*Cucumis sativus* L.) root exudates along with their germination stimulation activities toward *Orobanche minor* and *Phelipanche ramosa* seeds are described (Khetkam et al., 2014).

2-1. Materials and Methods

Instruments

Nuclear Magnetic Resonance (NMR) spectra

^{1}H and ^{13}C NMR spectra were recorded on a JEOL JMN-ECA-500 spectrometer in $CDCl_3$. The residual nondeuterated solvent signals at δ 7.26 and 77.0 ppm were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively.

Circular Dichroism (CD) spectra

CD spectra were recorded with a JASCO J-720 W spectropolarimeter in MeCN.

High Performance Liquid Chromatography (HPLC)

HPLC analysis and purification were performed on a Hitachi L-2310 low-pressure gradient system equipped with UV and PDA detectors.

GC–MS spectra

GC–MS spectra were obtained with a JOEL JMS-Q1000GC/K9 on a DB-5 (J&W Scientific, Agilent) capillary column (5 m × 0.25 μm) using a He as carrier gas (3 mL/min). Samples were introduced in a splitless mode. Column temperature was kept at 130°C for the first 1.5 min, elevated to 270°C with a 16°C/min gradient and kept at 270°C for 5 min.

High-resolution mass spectra

High-resolution mass spectrum was obtained with an Agilent 6250 Q-TOF mass spectrometer equipped with an ESI source.

LC–MS/MS spectra

HPLC separation was performed with a U980 HPLC instrument (JASCO, Tokyo, Japan) equipped with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 mm, 5 μm). The mobile phase was a gradient system of MeOH–water initially 60:40, and was changed to 100:0 at 30 min after injection. The flow rate was 0.2 mL/min, and the column temperature was set to 40°C. Mass spectrometry was obtained with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source. Nitrogen was used as the drying and nebulizing gas generated from pressurized air in an N2G nitrogen generator (Parker–Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approximately 100 L/h, and the desolvation gas flow to 500 L/h. The interface and source temperatures were set to 400 and 150°C, respectively. The capillary and cone voltages were adjusted to each molecule and to the positive ionization mode. MS/MS experiments were performed by using argon as the collision gas and the collision energy was set to 16 eV. The collision gas pressure was 0.15 Pa. Data acquisition and analysis were conducted with the MassLynx software (ver. 4.1).

Chemicals

The optically pure stereoisomers of orobanchol were generous gifts of Prof. Kohki Akiyama (Osaka Prefecture University, Japan). GR24 was provided by Prof. Tadao Asami (The University of Tokyo). Column chromatography (CC) was performed on silica gel Wako gel C-300 (Wako Pure Chemical Industries, Japan). The other chemicals of analytical grade and HPLC solvents were purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

Source of seeds

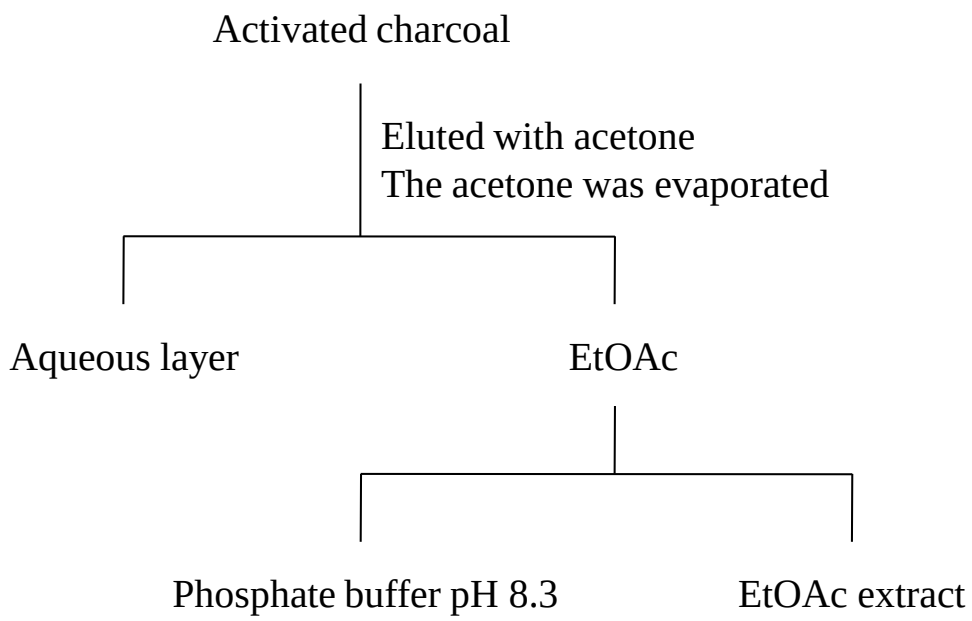
Orobanche minor Sm. (clover broomrape) seeds were collected from mature plants that were parasites of red clover (*Trifolium pratense*) grown in the Kinu basin of Tochigi Prefecture, Japan. *Phelipanche ramosa* seeds (pathotype 1, parasite of *Brassica napus*) were kindly provided by Prof. Philippe Delavault (University of Nantes, France). Seeds of cucumber (*Cucumis sativus* L. cv. Aonagakei-Jibai) were obtained from a local market.

Collection of root exudates

The cucumber seeds were germinated on moistened vermiculite in plastic containers (28.5 × 23.5 × 11 cm, W × L × H) for 2 weeks maintained at 23–27ºC under natural daylight conditions. The plants were watered with tap water when needed. Seedlings were transferred to a larger container (53.5 × 33.5 × 14 cm, W ×

L × H) containing 20 L of tap water as a culture medium. Each container containing about 50 seedlings was placed in a growth room maintained at 23–27ºC under natural daylight conditions as shown in Fig. 2-1. Root exudates released into the culture medium were adsorbed on activated charcoal (4 g × 2, for 20 L) using two water circulation pumps. The plants were grown for 5 weeks and the culture medium and activated charcoal were exchanged every 3–4 days. The root exudates absorbed on charcoal (8 g) were eluted with acetone (400 mL). After evaporation of the acetone *in vacuo*, the aqueous residue (*ca*. 70 mL) was extracted with EtOAc (3 × 70 mL). The EtOAc extracts were combined, washed with 0.2 M K_2HPO_4 (100 mL, pH 8.3), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The concentrated samples were kept at 4°C until use. The extraction sequence is shown in Scheme 2-1.

Fig. 2-1. Cucumber plants were grown in a hydroponic system.



Scheme 2-1. Extraction of cucumber root exudates.

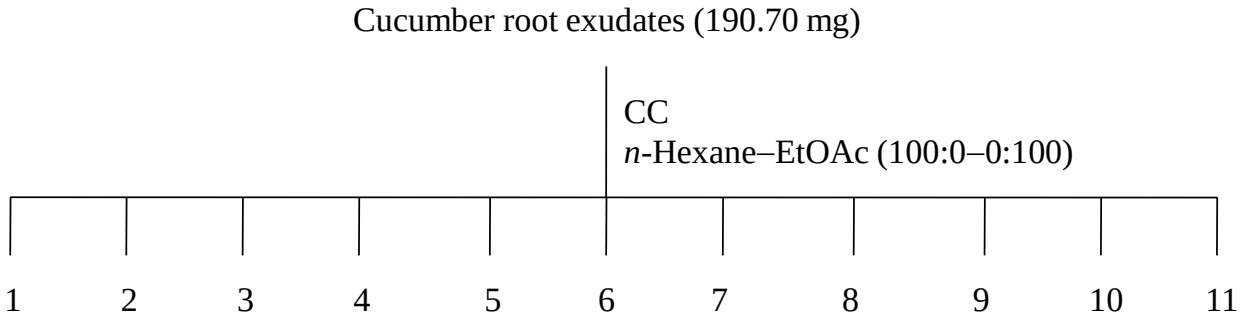
Seed germination assay

The seeds of parasites (*O. minor* and *P. ramosa*) were surface-sterilized in 1% NaClO containing 0.1% Tween-20 for 5 min, rinsed with sterile Milli-Q water and air-dried. The seeds about 30 each, were sown on 5-mm glass fiber disks (Whatman GF/A) and about 90 disks were placed in a 9-cm petri dish lined with a sheet of filter paper (No. 2, Advantec, Tokyo, Japan) moistened with 2.7 mL of sterile Milli-Q water. The petri dished were sealed with parafilm and incubated in the dark at 23°C for 7 days. Three disks carrying the conditioned seeds were then transferred to a 5-cm sterile petri dish lined with filter paper and treated with 650 μL of test solution. Each test solution, unless otherwise mentioned, contained 0.1% MeCN. The petri dishes were sealed, wrapped in aluminum foil, and placed in the

dark at 23°C for 4–5 days. Seeds treated with or without GR24 (10^{-6} M) were always included as positive and negative controls. Seeds were considered germinated when the radical protruded through the seed coat.

Isolation of 7α-hydroxyorobanchyl acetate (26) and 7β-hydroxyorobanchyl acetate (27)

The crude cucumber root exudates (190.70 mg) was subjected to column chromatography over silica gel (40 g, Wako gel C-300) using a gradient system of *n*-hexane–EtOAc (100:0–0:100) as eluent to obtain 11 fractions (Scheme 2-2). All fractions were concentrated and analyzed by LC–MS/MS.



Fraction 1 (15.61 mg) Fraction 2 (27.90 mg) Fraction 3 (20.62 mg)

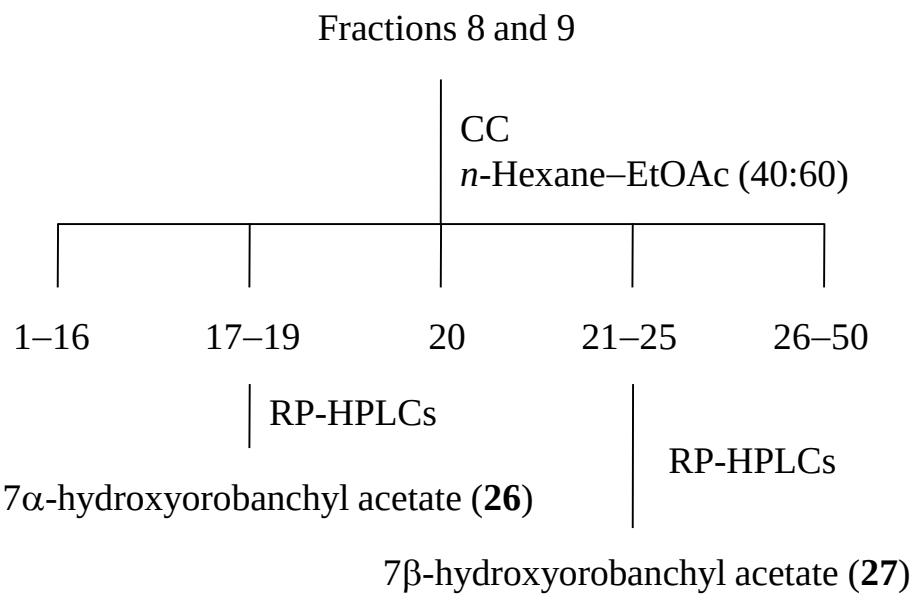
Fraction 4 (24.95 mg) Fraction 5 (26.77 mg) Fraction 6 (19.81 mg)

Fraction 7 (12.90 mg) Fraction 8 (8.23 mg) Fraction 9 (5.12 mg)

Fraction 10 (5.03 mg) Fraction 11 (4.84 mg)

Scheme 2-2. Purification of cucumber root exudates by silica gel CC.

Fractions 8 and 9 containing two novel SLs were combined (13.35 mg) and then subjected to column chromatography over silica gel (6 g) using an isocratic system of *n*-hexane–EtOAc (40:60, v/v) as the eluting solvent. Fractions were collected every 10 mL as shown in Scheme 2-3. Fractions 17–19 and fractions 21–25 were found to contain novel germination stimulants **26** and **27**, respectively, by LC–MS/MS and GC–MS analyses. The fractions 17–19 were combined (2.17 mg) and was further purified by HPLC on an ODS (C_{18}) column (Mightysil RP-18, 4.6 × 250 mm, 5 μm; Kanto Chemicals, Japan) using a gradient system of MeCN–H_2O (10:90–60:40 over 50 min) as the eluting solvent. The flow rate was 0.8 mL/min, and the column temperature was set to 30°C. Detection was carried out at 238 nm. The active fraction eluted as a single peak at the retention time of 21.4 min was collected and further purified by HPLC using isocratic system of MeCN–H_2O (70:30, v/v) on a Develosil ODS-CN column (4.6 × 250 mm, 5 μm; Nomura Chemicals, Japan) at a flow rate of 0.8 mL/min to afford compound **26** (0.83 mg, t_R 27.7 min). The fractions 21–25 were combined (1.10 mg) and was purified in a similar manner as described above to give compound **27** (0.31 mg) with the retention times of 20.1 and 26.2 min in the ODS-HPLC and ODS-CN HPLC, respectively.



Scheme 2-3. Purification of novel stimulants (**26**, **27**) by silica gel CC and RP-HPLCs.

7α-Hydroxyorobanchyl acetate (26)

CD (*c*0.0005, MeCN) λ_{max} ($\Delta\varepsilon$) nm: 218 (76.10), 254 (–5.61). GC–MS,70eV, *m/z* (rel. int): 404 $[M]^+$ (1), 362 (2), 344 (4), 247 (22), 97 (100). HR–TOF–MS *m/z*: 405.1564 $[M + H]^+$ (calcd. for $C_{21}H_{25}O_8$, *m/z*: 405.1549). NMR spectroscopic data are given in Table 2-1.

7β-Hydroxyorobanchyl acetate (27)

CD (*c*0.0010, MeCN) λ_{max} ($\Delta\varepsilon$) nm: 218 (38.09), 254 (–4.11). GC–MS, 70 eV, *m/z* (rel. int): 404 $[M]^+$ (1), 362 (2), 344 (5), 247 (23), 97 (100). HR–TOF–MS *m/z*: 405.1542 $[M + H]^+$ (calcd. for $C_{21}H_{25}O_8$, *m/z*: 405.1549). NMR spectroscopic data are given in Table 2-2.

Table 2-1. NMR spectroscopic data for 7α-hydroxyorobanchyl acetate (**26**) ($CDCl_3$).

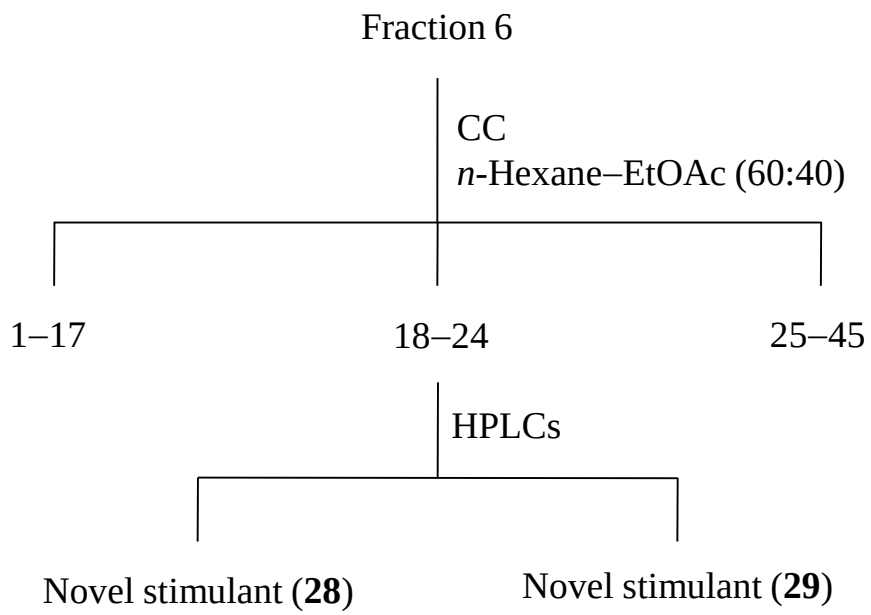
Position	δ_H (mult., *J*Hz)	δ_C	DEPT and HMQC	^1H–^1HCOSY	HMBC	NOESY
2		169.7	C			
3		110.1	C			
3a	3.50 (*ddd*, 5.9, 2.0, 1.8)	45.8	C	H-6′, 8b		H-8b
4	5.72 (*bs*)	82.2	CH	H-8b		
4a		139.4	C			
5	1.97–2.11 (*m*)	21.8	CH_2			
6	1.78–1.85 (*m*)	26.4	CH_2	H-7		
7	3.54 (*dd*, 8.2, 3.2)	75.3	CH	H-6		H-9
8		37.3	C			
8a		145.7	C			
8b	5.60 (*bd*,6.0)	85.3	CH	H-3a, 4		H-9, 3a
9	1.24 (*s*)	21.0	CH_3		C-7, 8, 8a, 10	H-7, 8b
10	1.12 (*s*)	24.1	CH_3		C-7, 8, 8a, 9	
2′	6.16 (*t*, 1.4)	99.8	CH	H-3′, 7′	C- 6′	H-6′
3′	6.95 (*t*, 1.4)	140.8	CH	H-2′, 7′		H-2′
4′		136.3	C			
5′		170.2	C			
6′	7.48 (*d*, 2.3)	150.8	CH	H-3a	C-2, 2′, 3, 3a	H-2′
7′	2.03 (*t*,1.4)	10.8	CH_3	H-2′, 3′	C-3′, 4′, 5′	
1′′		170.3	C			
2′′	2.04 (*s*)	20.7	CH_3			

Table 2-2. NMR spectroscopic data for 7β-hydroxyorobanchyl acetate (**27**) ($CDCl_3$).

Position	δ_H (mult., *J*Hz)	δ_C	DEPT and HMQC	^1H–^1HCOSY	HMBC	NOESY
2		169.8	C			
3		110.3	C			
3a	3.50 (*ddd*, 5.9, 2.0, 1.8)	45.4	C	H-4, 6′, 8b		H-8b
4	5.75 (*bs*)	82.5	CH	H-3a, 8b		
4a		139.3	C			
5	1.99–2.15 (*m*)	19.7	CH_2			
6	1.77–1.95 (*m*)	25.4	CH_2	H-7		
7	3.62 (*bd*, 5.0)	74.8	CH	H-6		H-10
8		37.3	C			
8a		144.4	C			
8b	5.60 (*bd*, 6.0)	85.6	CH	H-3a, 4		H-9, 3a
9	1.21 (*s*)	21.4	CH_3		C-7, 8, 8a, 10	H-8b
10	1.16 (*s*)	27.2	CH_3		C-7, 8, 8a, 9	H-7
2′	6.16 (*t*, 0.9)	99.8	CH	H-3′, 7′	C-6′	H-6′
3′	6.95 (*t*,0.9)	140.8	CH	H-2′, 7′		
4′		136.2	C			
5′		170.2	C			
6′	7.48 (*d*, 1.8)	150.8	CH	H-3a	C-2, 2′, 3, 3a	H-2′
7′	2.03 (*t*, 1.4)	10.8	CH_3	H-2′, 3′	C-3′, 4′, 5′	
1′′		170.4	C			
2′′	2.04 (*s*)	21.0	CH_3			

Detection of 7α-hydroxyorobanchol (24) and 7β-hydroxyorobanchol (25), and isolation of two novel germination stimulants (28, 29)

Fraction 6 (19.81 mg) from the first silica gel CC was subjected to silica gel CC (6 g) using an isocratic system of *n*-hexane–EtOAc (60:40, v/v) as eluent. Fractions were collected every 10 mL to give 45 fractions. All fractions were subjected to LC–MS/MS analysis. The fractions 18–24 (5.45 mg) containing novel stimulants were combined and was purified by HPLC on an ODS column (Mightysil RP-18, 10 × 250 mm, 10 µm; Kanto Chemicals, Japan) with an isocratic system of MeCN–H_2O (50:50, v/v) at a flow rate of 3.0 mL/min. Detection was performed at 240 nm. The novel stimulants eluted as a single peak at 15.6 min was collected, and was further purified by HPLC on a silica gel column (Inertsil SIL 100A, 4.6 × 250 mm, 3 µm; GL Sciences Inc., Japan) using an isocratic system of *n*-hexane–EtOAc (55:45, v/v) at flow rate of 1 mL/min to give pure compounds **28** (0.15 mg, t_R 14.3 min) and **29** (< 0.1 mg, t_R 16.3 min).



Scheme 2-4. Purification of novel stimulants (**28**, **29**) by silica gel CC and HPLCs.

Novel stimulant (28)

^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.24 (3H, s), 1.31 (3H, s), 1.50–1.60 (2H, m), 1.69–1.75 (2H, m), 1.99 (3H, t, *J* = 1.4 Hz), 2.34 (1H, dt, *J* = 16.0, 5.0 Hz), 2.42 (1H, dt, *J* = 16.0, 5.0 Hz), 3.76 (3H, s), 5.78 (1H, s), 6.02 (1H, br s), 6.86 (1H, t, *J* = 1.4 Hz), 7.62 (1H, s). ESI–MS *m/z*: 391 $[M + H]^+$, 413 $[M + Na]^+$. GC–MS, 70 eV, *m/z* (rel. int): 390 (1), 293 (66), 261 (100), 233 (60), 97 (34). HR–TOF–MS *m/z*: 391.1396 $[M + H]^+$ (calcd. for $C_{20}H_{23}O_8$, *m/z*: 391.1387).

Novel stimulant (29)

^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.20 (3H, s), 1.31 (3H, s), 1.59–1.65 (2H, m), 1.66–1.77 (2H, m), 1.99 (3H, t, *J* = 1.4 Hz), 2.19 (1H, dt, *J* = 16.0, 5.0 Hz), 2.44 (1H, dt, *J* = 16.0, 5.0 Hz), 3.77 (3H, s), 5.98 (1H, s), 6.00 (1H, t, *J* = 1.0 Hz), 6.86 (1H, t, *J* = 1.4 Hz), 7.61 (1H, s). ESI–MS *m/z*: 391 $[M + H]^+$, 413 $[M + Na]^+$. GC–MS, 70 eV, *m/z* (rel. int): 390 (1), 293 (53), 261 (100), 233 (67), 97 (38). HR–TOF–MS *m/z*: 391.1392 $[M + H]^+$ (calcd. for $C_{20}H_{23}O_8$, *m/z*: 391.1387).

2-2. Results and Discussion

2-2-1. Characterization of strigolactones produced by cucumber

The cucumber plants had been grown by hydroponic system for 5 weeks. The root exudates released into culture media was collected by activated charcoal (Akiyama et al., 2005). After elution of activated charcoal by acetone, the acetone was evaporated and the aqueous residue was then partitioned by EtOAc to give a neutral crude root exudates. One ten-thousandth of this crude extract was analyzed by LC–MS/MS operated in the ESI positive mode. Four known SLs, 7-oxoorobanchyl acetate (**16**), orobanchyl acetate (**12**), orobanchol (**11**), and 4-deoxyorobanchol (**10**) were detected by monitoring the transitions of *m/z* 425 > 268, 411 > 254, 369 > 272, and 353 > 256, respectively as shown in Figs. 2-2 and 2-3A. The identities of these SLs were confirmed with co-chromatography in LC–MS/MS using authentic SL standards. One hundred-thousandth of the crude extract was subjected to RP-HPLC operated under the same conditions as for LC–MS/MS analyses and the fractions collected every minute were examined for *O. minor* seed germination stimulation. The distribution of germination stimulation activities toward *O. minor* seeds suggested that the root exudates contained several novel germination stimulants including hydroxyorobanchyl acetate isomers in addition to these known SLs (Fig. 2-3B). In Fig. 2-3B, 4-deoxyorobanchol was eluted slightly earlier as compared to that in Fig. 2-3A.

The novel stimulants were detected in the fractions 5, 6, and 9, which induced high *O. minor* seed germination (> 80%). The sodium adduct ions at *m/z*

427, 413, and 397 were detected in LC–MS/MS for fractions 5, 6, and 9, respectively (Fig. 2-4). The molecular ion at *m/z* 390 was detected for fraction 6 by GC–MS analysis (Fig. 2-5).

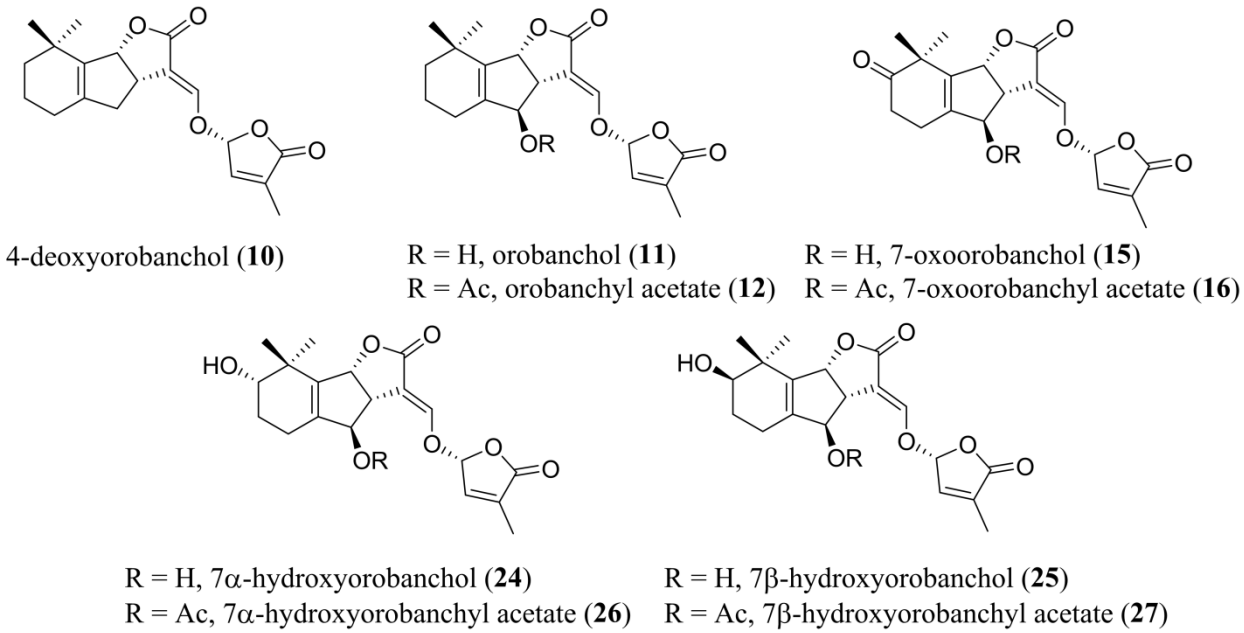


Fig. 2-2. Chemical structures of SLs produced by cucumber plants.

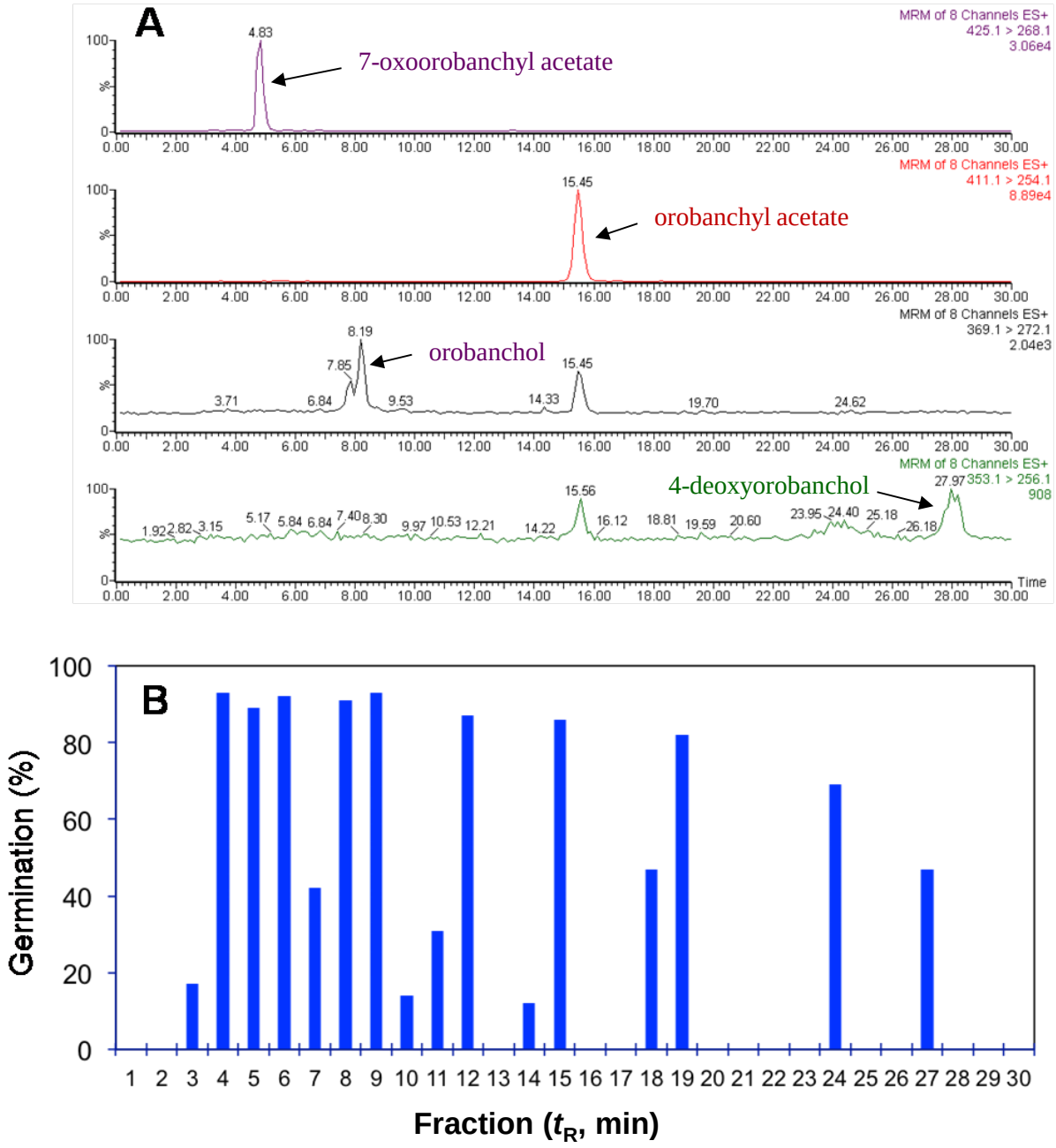


Fig. 2-3. LC–MS/MS (MRM) chromatograms of cucumber root exudates (A). The transitions of *m/z* 425 > 268, 411 > 254, 369 > 272, and 353 > 256 were monitored for 7-oxoorobanchyl acetate (**16**), orobanchyl acetate (**12**), orobanchol (**11**), and 4-deoxyorobanchol (**10**), respectively. Distribution of germination stimulation activity after reverse phase HPLC separation of the root exudates of cucumber (B). The fractions collected every minute were conducted to germination stimulation on *O. minor* seeds.

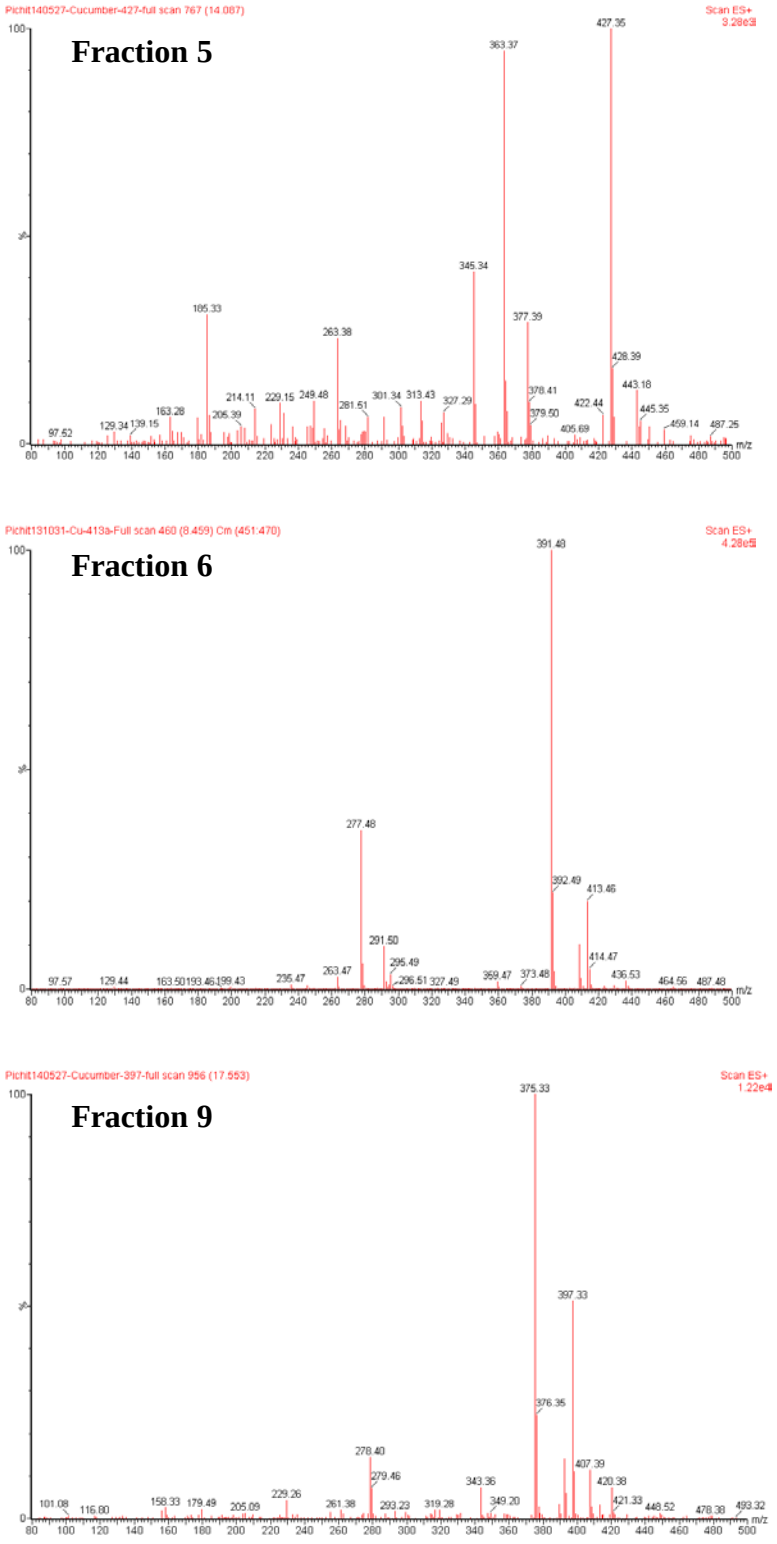


Fig. 2-4. ESI–MS spectra of novel stimulants found in the fractions 5, 6, and 9.

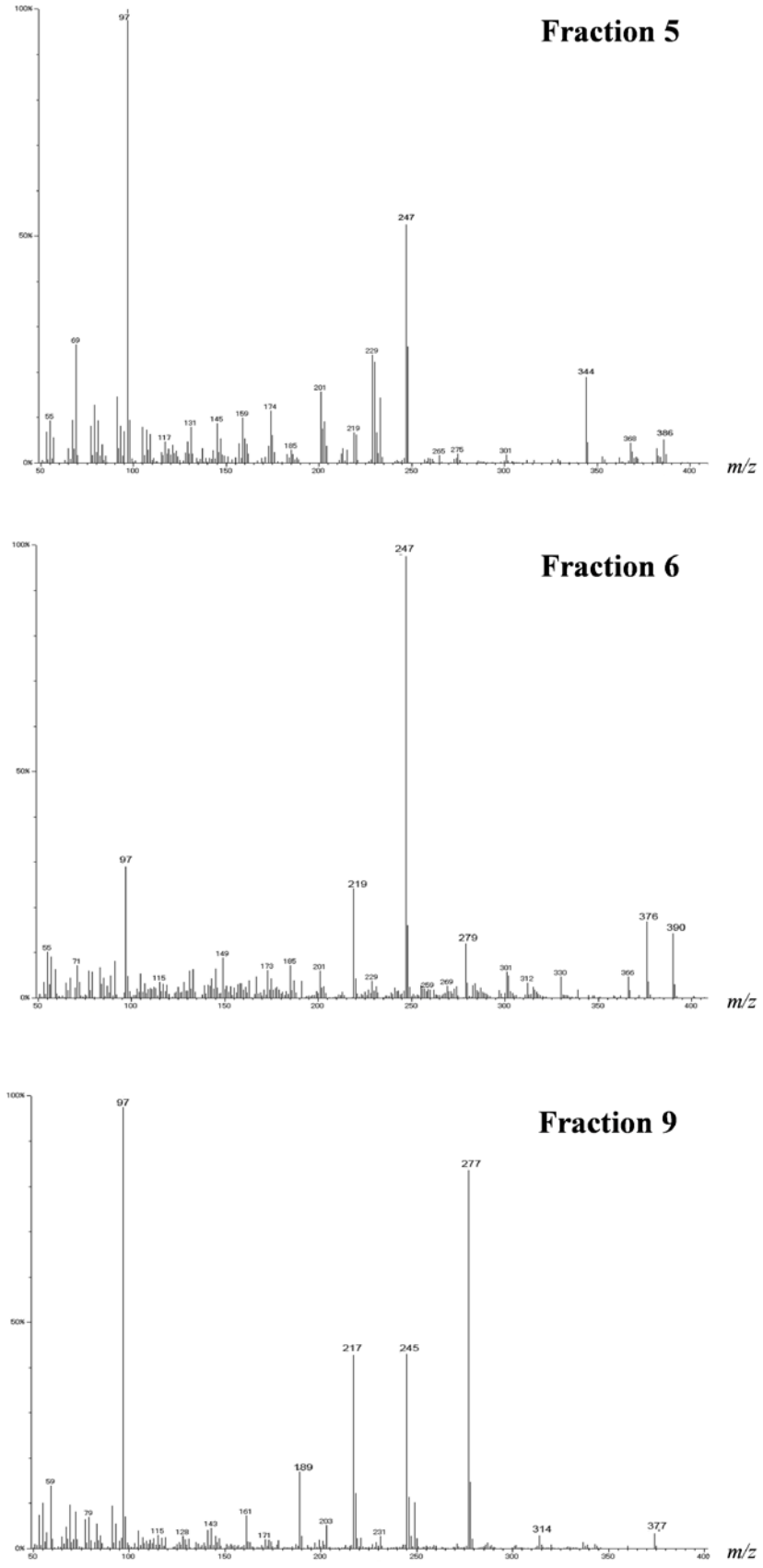


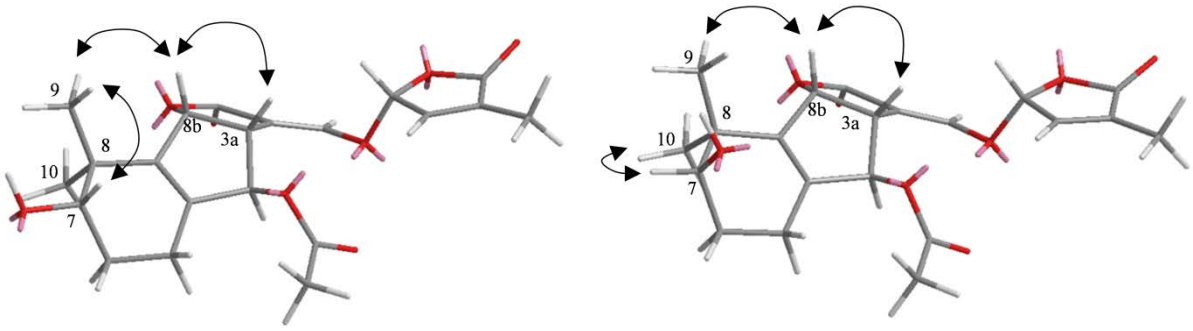
Fig. 2-5. GC–MS spectra of novel stimulants found in the fractions 5, 6, and 9.

2-2-2. Isolation and structural elucidation of 7α-hydroxyorobanchyl acetate (26) and 7β-hydroxyorobanchyl acetate (27)

The crude extract of cucumber root exudates was subjected to silica gel column chromatography using *n*-hexane–EtOAc as eluting solvent. All of fractions were examined for their germination stimulation activity on *O. minor* seeds. The active compounds were found to be eluted in the 40–90% EtOAc fractions. By LC–MS/MS analysis, six known SLs, orobanchol (**11**), orobanchyl acetate (**12**), 7-oxoorobanchol (**15**), 7-oxoorobanchyl acetate (**16**), and 4-deoxyorobanchol (**10**) were detected. In addition, two novel SLs were detected from the 70–80% EtOAc fractions. These fractions were combined and further purified by silica gel column chromatography and HPLCs to obtain two novel SLs, compounds **26** and **27**. These compounds were subjected to spectroscopic analyses for structural determination.

The molecular formulae of both compounds **26** and **27** were established as $C_{21}H_{24}O_8$ by HR–TOF–MS analyses. In addition, this molecular formula was confirmed by the presence of sodium adduct ion at *m/z* 427 in LC–MS and molecular ion at *m/z* 404 in GC–MS indicating that these compounds were hydroxyorobanchyl acetate isomers. The ^{1}H and ^{13}C NMR spectra of compounds **26** and **27** were very similar to those of orobanchyl acetate (**12**) (Xie et al., 2008, Matsuura et al., 2008) and especially to those of 7-oxoorobanchyl acetate (**16**) (Xie et al., 2009) indicating that these molecules contained the common structural

feature for the known SLs (Yoneyama et al., 2013). Assignments of the ^{1}H and ^{13}C NMR data were achieved by 2D NMR (COSY, DEPT, HMQC, and HMBC) techniques (Tables 2-1 and 2-2). The doublet of doublet signal at 3.54 ppm (J = 8.2, 3.2 Hz) of compound **26** indicated the presence of a hydroxyl group at C-7. The absence of signals of methylene protons at C-7 also supported the presence of a 7-hydroxyl group. The H-7 signal of compound **26** shows NOE correlations with 9-Me, and the 9-Me signal with H-7 and H-8b, indicating that relative stereochemistry of the 7-hydroxyl group in compound **26** is *cis* to the C ring. By contrast, the NOE correlation between H-7 and 10-Me in compound **27** demonstrates that the relative stereochemistry of the hydroxyl group is opposite to that in compound **26** as shown in Fig. 2-6. Therefore, compounds **26** and **27** are diastereomers with different stereochemistry of the 7-hydroxyl group.



(26) **(27)**

Fig. 2-6. Selected NOE correlations of 7α-hydroxyorobanchyl acetate (**26**) and 7β-hydroxyorobanchyl acetate (**27**).

The CD spectra of compounds **26** and **27** showed a positive and negative Cotton effect at around 218 nm and 254 nm, respectively (Fig. 2-7) which were similar to that of orobanchol-type SLs suggesting that compounds **26** and **27** have a 2'(*R*) configuration carrying an α-oriented C-ring. Accordingly, compounds **26** and **27** were determined to be 7α-hydroxyorobanchyl acetate and 7β-hydroxyorobanchyl acetate, respectively.

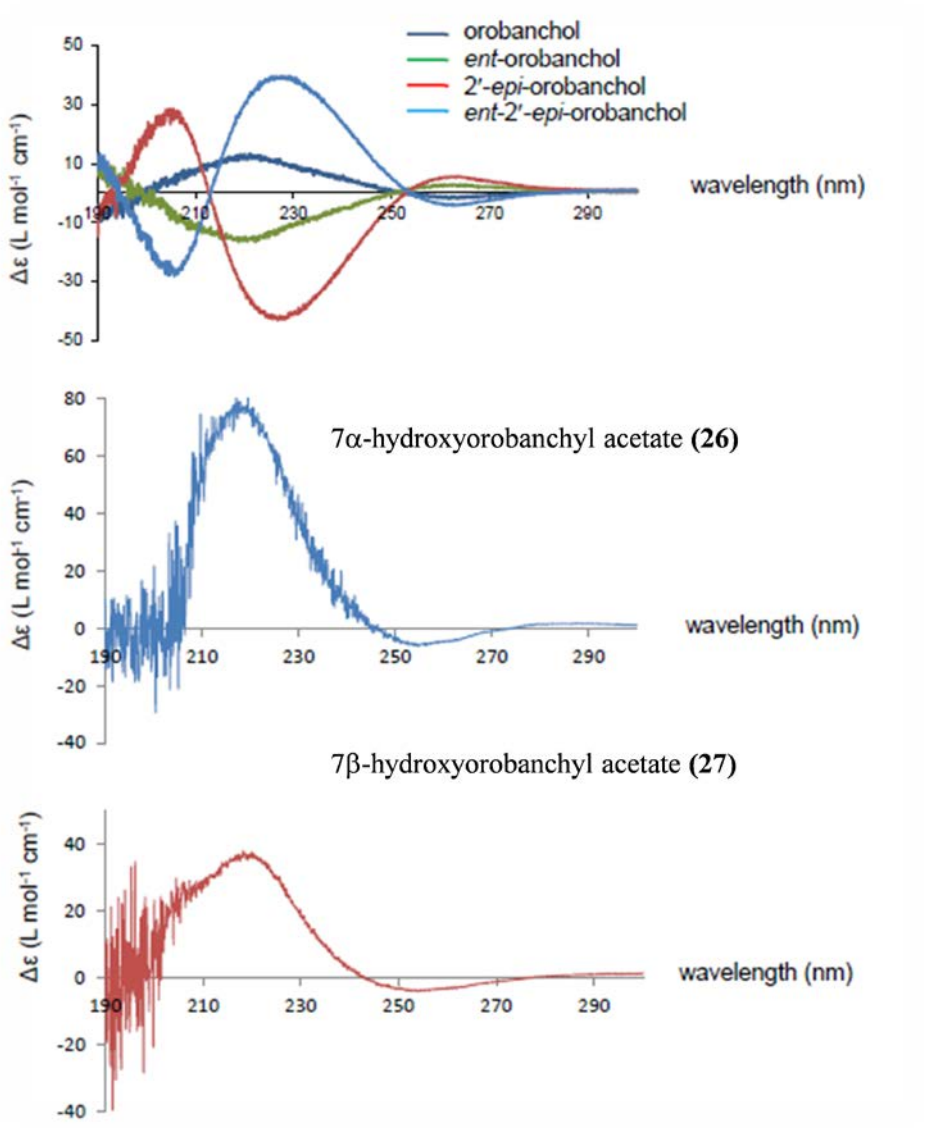


Fig. 2-7. CD spectra of synthetic standards of orobanchol, *ent*-orobanchol, 2′-*epi*-orobanchol and *ent*-2′-*epi*-orobanchol, and 7α-hydroxyorobanchyl acetate (**26**) and 7β-hydroxyorobanchyl acetate (**27**) isolated from cucumber root exudates.

2-2-3. Detection of 7α-hydroxyorobanchol (24), 7β-hydroxyorobanchol (25), and other minor germination stimulants

In the 90% EtOAc fraction of the first silica gel CC, which was moderately active in *O. minor* seed germination assay, two novel germination stimulants were detected by LC–MS/MS monitoring the transition of *m/z* 385 > 288, indicating that these were hydroxyorobanchol isomers. Therefore this fraction was subjected to purification by RP-HPLCs but we could obtain only trace amount (< 0.1 μg) of an inseparable mixture of two isomeric compounds which decomposed gradually even in solutions with aprotic organic solvents. The LC–MS and GC–MS analytical data of these compounds strongly suggested that these compounds are 7α-hydroxyorobanchol (**24**) and 7β-hydroxyorobanchol (**25**) (Figs. 2-8 and 2-9). Indeed, acetylation of these compounds afforded monoacetyl esters which showed the same retention times and spectra in the LC–MS/MS and GC–MS analytical data with those of compounds **26** and **27**.

In addition to these SLs, three SL-like germination stimulants were detected by LC–MS/MS as described earlier (Fig. 2-4). In the mass spectra, they showed fragmentation patterns typical for SLs such as a base peak at *m/z* 97 in GC–MS (Fig. 2-5). Among these stimulants, the one detected in fraction 6 was subjected to further purifications, as its amount seemed to be larger than those of the other two.

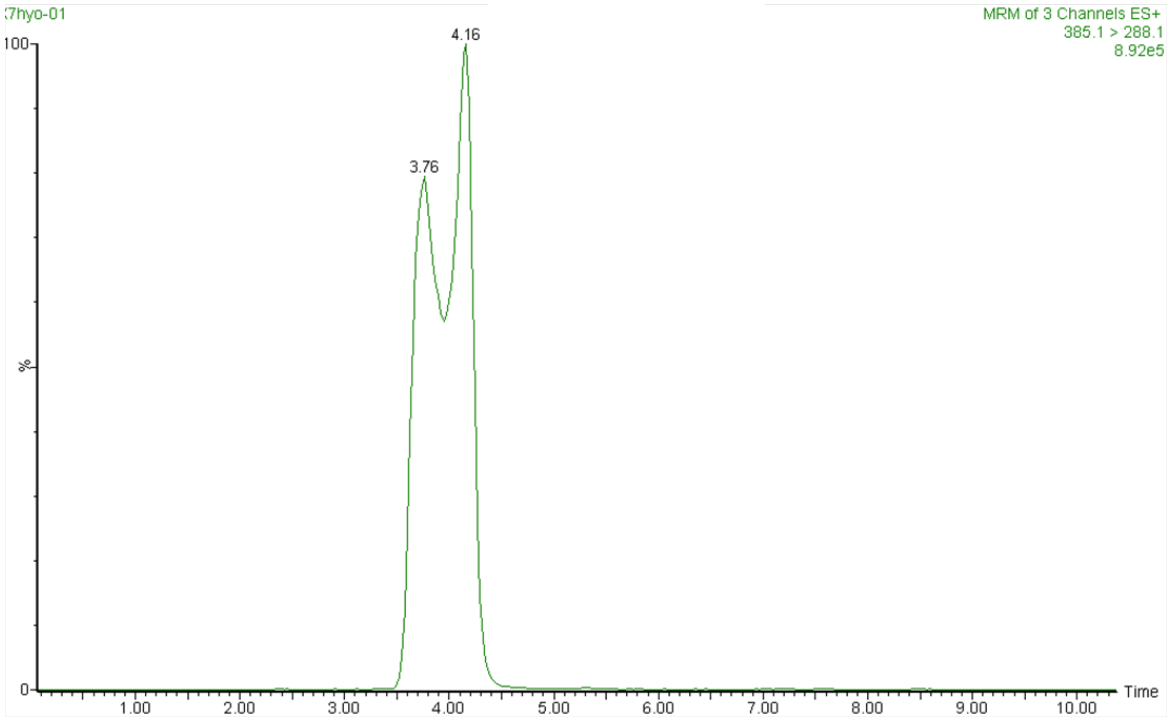


Fig. 2-8. LC–MS/MS chromatogram of the mixture of 7α-hydroxyorobanchol (**24**) and 7β-hydroxyorobanchol (**25**). The transition of *m/z* 385 > 288 was monitored.

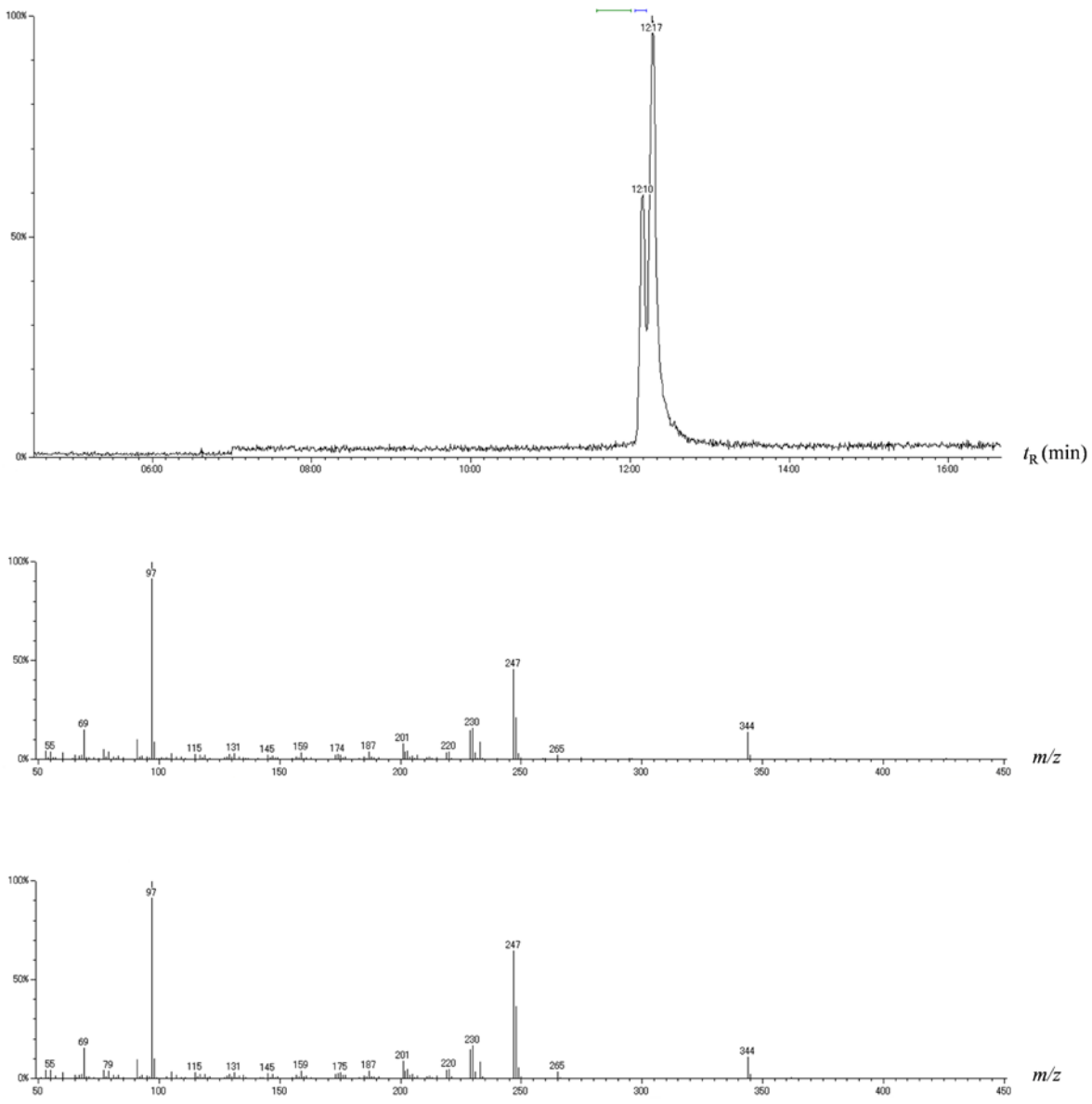


Fig. 2-9. GC–MS spectra of 7α-hydroxyorobanchol (**24**) and 7β-hydroxyorobanchol (**25**) found in cucumber root exudates.

2-2-4. Isolation of novel SL-like germination stimulants (28, 29)

The germination stimulant in the 50% EtOAc fraction, fraction 6, was found to be a mixture of two isomers, and purifications by ODS and silica gel HPLCs afforded compounds **28** and **29**. The pure compounds were subjected to spectroscopic analyses for structural determination. Unfortunately, the quantities

were not enough for ^{13}C and 2D NMR experiments. The molecular formulae of compounds **28** and **29** were established as $C_{20}H_{22}O_8$ by HR–TOF–MS, LC–MS, and GC–MS analyses. The ^{1}H NMR spectra of both compounds are very similar to each other and the signals at δ1.99 (3H, t, *J* = 1.4 Hz), 6.02 (1H, t, *J* = 1.0 Hz), 6.86 (1H, t, *J* = 1.4 Hz) and 7.62 (1H, s) indicate the presence of the D ring. In addition, the absence of typical B-C ring signals corresponding to H-3a, H-4 and H-8b in SLs and the presence of methyl ester signal at 3.75 ppm suggest that these compounds are isomers containing the A and D rings of typical SLs but lacking the B and C rings as shown in Fig. 2-10. These compounds may be derived from carlactone (**23**), a biosynthetic intermediate for SLs. Since all natural SLs identified in plant root exudates have a 2′*R* configuration (Yoneyama et al., 2013) and also the same stereochemistry has been confirmed for C-11 of carlactone (Seto et al., 2014), the stereochemistry at C-11 of compounds **28** and **29** would be *R*-configuration.

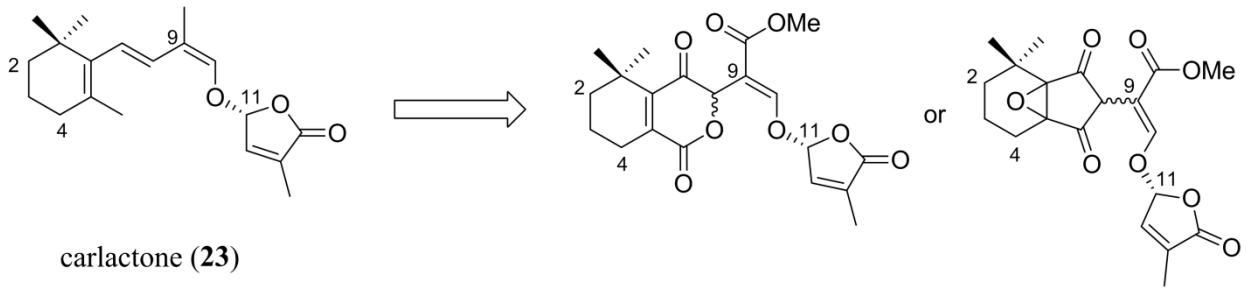


Fig. 2-10. The tentative structures of novel germination stimulants **28** and **29** isolated from cucumber root exudates.

2-2-5. Germination stimulation activities of 7α-hydroxyorobanchyl acetate (26) and 7β-hydroxyorobanchyl acetate (27) on *O. minor* and *P. ramosa* seeds

The germination stimulation activities of 7α-hydroxyorobanchyl acetate (**26**), 7β-hydroxyorobanchyl acetate (**27**), 7-oxoorobanchyl acetate (**16**), and orobanchyl acetate (**12**) toward *O. minor* and *P. ramosa* seeds are shown in Fig. 2-11. All of these SLs induced similar levels of high germination (~80%) of *O. minor* seeds at 0.1 nM. These results indicate that the introduction of a carbonyl or hydroxyl group at C-7 does not affect germination stimulation activity toward *O. minor* seeds. By contrast, 7β-hydroxyorobanchyl acetate (**27**) was found to be a highly potent germination stimulant for *P. ramosa* seeds and inducing more than 50% germination at 10 pM, suggesting that the β-hydroxyl group at C-7 may be involved in the interaction between the stimulant and its receptor site in the seed of this parasitic weed.

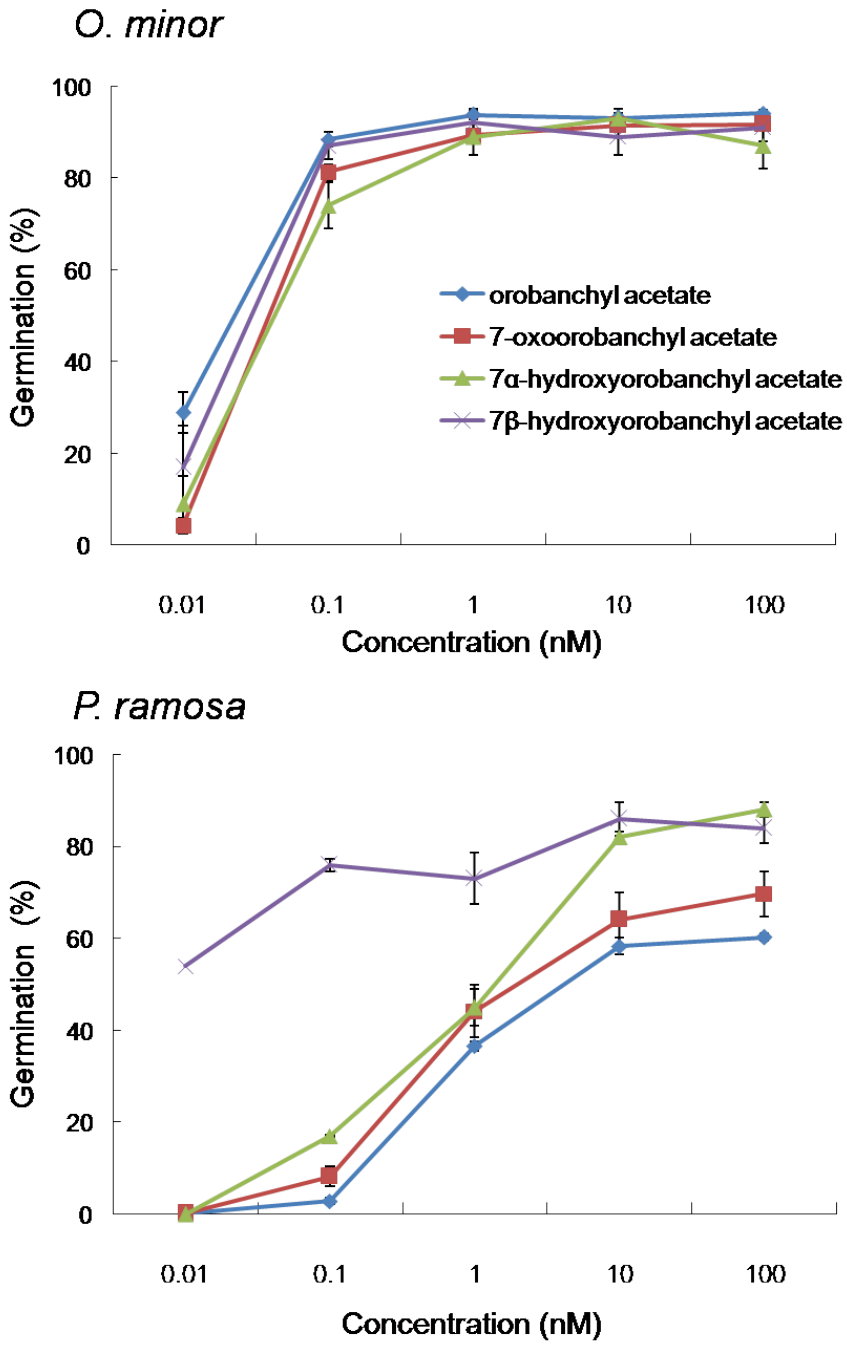


Fig. 2-11. Germination stimulation activity of SLs found in the cucumber root exudates toward *O. minor* and *P. ramosa* seeds. Each value is presented as a mean ± SE (n = 3).

Chapter 3. Isolation and Characterization of Germination Stimulants Produced by Maize

Maize (*Zea mays*) is a major host of the root parasitic weeds, *Striga hermonthica* and *S. asiatica*, and has been reported to produce several SLs. Among known SLs, strigol (**2**) was isolated as the major *Striga* seed germination stimulant from maize root exudates (Siame et al., 1993). In addition to this SL, sorgomol (**5**) and 5-deoxystrigol (**1**) were found from maize root exudates (Awad et al., 2006). Recently, Jamil et al. (2012) have reported the detection of novel germination stimulants called SL1 and SL2 from maize root exudates by LC–MS/MS analysis. SL1 and SL2 were described to have the molecular weight of 348 and 376, respectively (Jamil et al. 2012). In this chapter, characterization and isolation of germination stimulants from maize root exudates are described.

3-1. Materials and Methods

Instruments

All instruments and analytic conditions were the same as described in Chapter 2, except that the LC–MS/MS instrument equipped with L-column2 ODS (C_{18}) column (CERI, Japan, 50 × 2.1 mm, 2 μm) was used. The mobile phase was a gradient system of MeOH–water initially 30:70, 45:55 at 3 min, 50:50 at 8 min, 70:30 at 12 min, 100:0 at 15 min.

Source of seeds

The source of *O. minor* seeds was the same as in Chapter 2.*Striga hermonthica* seeds were kindly provided by Prof. A. G. T. Babiker (Sudan University of Science and Technology, Sudan). Seeds of maize (*Zea mays* cv. Pioneer 2817) were purchased from a local market.

Collection of root exudates

The seeds of maize (*Zea mays* cv. Pioneer 2817) were sown in plastic pot and filled with autoclaved sand. The plant were grown in a growth room maintained at 23–27 °C under natural daylight conditions for 7 days. The plants were watered with tap water as required. The 250 seedlings were transferred to a plastic container (53.5 × 33.5 × 14 cm, W × L × H) containing 20 L of tap water and 10 mM $Ca(NO_3)_2$. Eight containers each containing 50 seedlings was placed in a growth room maintained at 23–27°C under natural daylight conditions as shown in

Fig. 3-1. Root exudates released into the culture medium were adsorbed on activated charcoal (4 g × 2, for 20 L) using two water circulation pumps. The plants were grown for 5 weeks and the culture medium and activated charcoal were replaced every 3–4 days. The root exudates absorbed on charcoal (64 g) were eluted with acetone (1,200 mL). After evaporation of the acetone in *vacuo*, the aqueous residue (*ca*. 200 mL) was extracted with EtOAc (3 × 200 mL). The EtOAc extracts were combined, washed with 0.2 M K_2HPO_4 (300 mL, pH 8.3), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The concentrated samples were kept at 4°C until use. The extraction sequence is shown in Scheme 3-1.

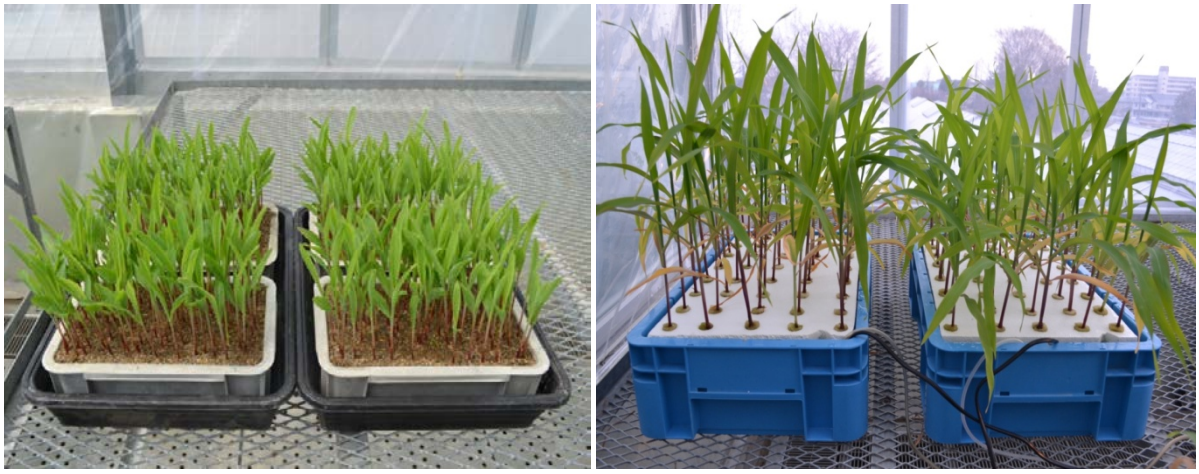


Fig. 3-1. Maize plants were grown in sand pot and the seedlings were then transferred to hydroponic system.

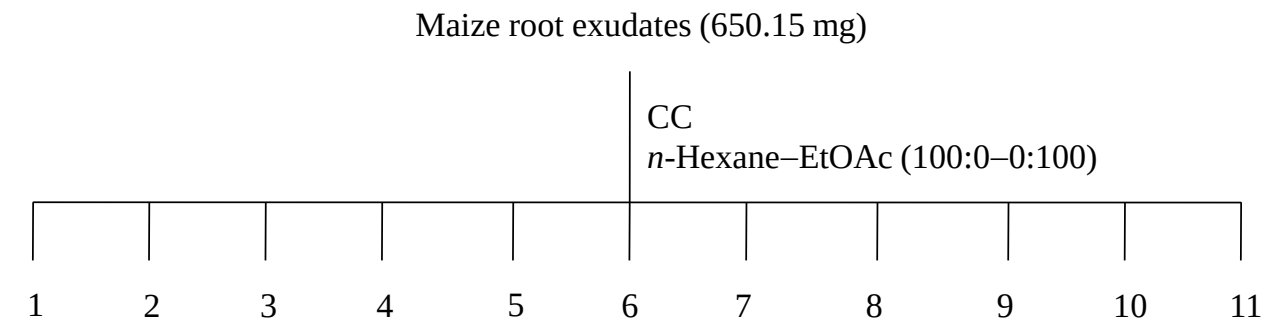
Seed germination assay

Germination tests were conducted as described in Chapter 2, except that the *S. hermonthica* seeds were conditioned in the dark at 30°C for 14 days and 30°C for 3 days after the seeds treated with the samples which the germination rates were determined.

Isolation and identification of novel germination stimulant from maize root exudates

The crude EtOAc extract of maize root exudates (650.15 mg) was subjected to silica gel column chromatography (65 g, Wako gel C-300) using a gradient system of *n*-hexane–EtOAc (100:0–0:100) as eluting solvent to give 11 fractions as shown in Scheme 3-1. All fractions were concentrated *in vacuo* and were examined for germination stimulant activity toward *O. minor* seeds as shown in Fig. 3-1.

Fraction 5 (64.4 mg) containing novel germination stimulant SL2 was subjected to column chromatography over silica gel (25 g) using an isocratic system of *n*-hexane–EtOAc (40:60, v/v) as eluting solvent. Fractions were collected every 10 mL as shown in Scheme 3-2.



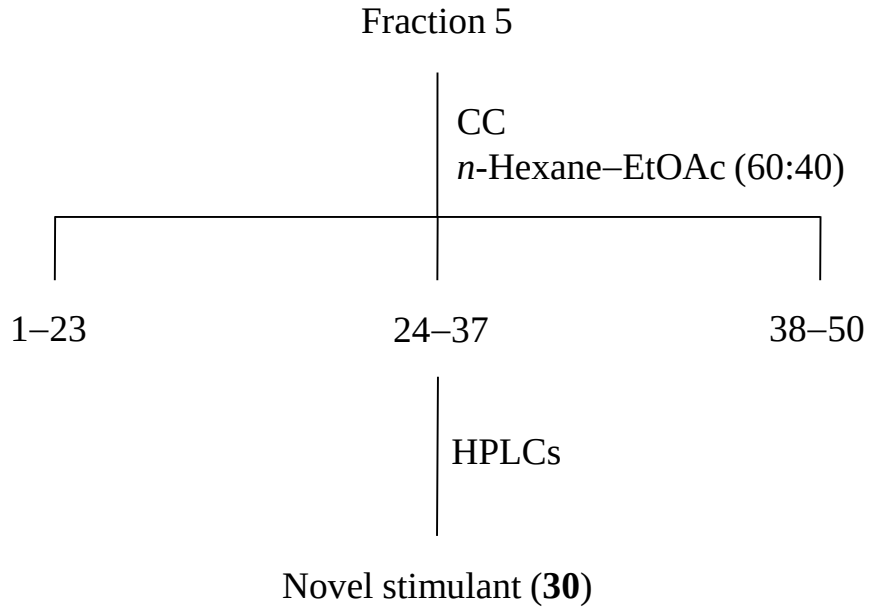
Fraction 1 (37.8 mg) Fraction 2 (67.7 mg) Fraction 3 (164.1 mg)

Fraction 4 (61.6 mg) Fraction 5 (64.4 mg) Fraction 6 (58.1 mg)

Fraction 7 (25.9 mg) Fraction 8 (19.0 mg) Fraction 9 (12.4 mg)

Fraction 10 (10.4 mg) Fraction 11 (8.2 mg)

Scheme 3-1. Purification of maize root exudates by silica gel CC.



Scheme 3-2. Purification of novel stimulant **30** by silica gel CC and HPLCs.

Fractions 24–37 were found to contain a novel germination stimulant **30** by LC–MS/MS and GC–MS analyses. The fractions 24–37 were combined (20.17 mg) and was further purified by HPLC on an ODS (C_{18}) column (Mightysil RP-18, 10 × 250 mm, 10 μm; Kanto Chemicals, Japan) using a gradient system of MeCN–H_2O (40:60–100:0 over 60 min) as eluting solvent. The flow rate was 3 mL/min, and the column temperature was set to 25°C. Detection was carried out at 238 nm. The active fraction eluted as the single peak at the retention time of 23.9 min was collected and further purified by HPLC using an isocratic system of MeCN–H_2O (70:30, v/v) on a Develosil ODS-CN column (4.6 × 250 mm, 5 μm; Nomura Chemicals, Japan) at a flow rate of 1 mL/min to obtain compound **30** (0.83 mg, t_R 24.5 min).

Novel stimulant (30)

^{1}H NMR (500 MHz, C_6D_6) δ: 0.67 (3H, s), 0.93 (3H, s), 1.19 (3H, t, *J* = 1.5 Hz), 1.57 (3H, s), 1.83 (1H, d, *J* = 13.2 Hz), 2.18 (1H, d, *J* = 13.2 Hz), 3.28 (3H, s), 4.66 (1H, quin, *J* = 1.2 Hz), 4.88 (1H, quin, *J* = 1.2 Hz), 5.14 (1H, s), 5.46 (1H, quin, *J* = 1.6 Hz), 6.89 (1H, d, *J* = 13.8 Hz), 7.25 (1H, d, *J* = 13.8 Hz), 7.39 (1H, s). ESI–MS *m/z*: 377 $[M + H]^+$, 399 $[M + Na]^+$. GC–MS, 70 eV, *m/z* (rel. int): 376 (30), 279 (10), 223 (31), 153 (100), 111 (87), 97 (27). HR–TOF–MS *m/z*: 377.1604 $[M + H]^+$ (calcd. for $C_{20}H_{25}O_7$, *m/z*: 377.1595).

3-2. Results and Discussion

3-2-1. Characterization of strigolactones and some other minor novel germination stimulants produced by maize

Maize plants were grown hydroponically for 5 weeks and the root exudates collected by using activated charcoal. One ten-thousandth of the crude EtOAc extract of root exudates was subjected to LC–MS/MS analysis. As shown in the Fig. 3-2, three known SLs, strigol (**2**), sorgomol (**5**) and orobanchol (**11**) were detected in the MRM monitoring transition of *m/z* 369 > 272, and 5-deoxystrigol (**1**) as a minor component in the transition of *m/z* 353 > 256. In addition to these SLs, three novel germination stimulants were detected in the transitions of *m/z* 371 > 274, 399 > 302, and 383 > 286 which were selected for SL1, SL2, and methoxy-5-deoxystrigol, respectively. In the MRM chromatogram, the two or three intense peaks appear in each channel monitoring novel stimulants suggesting that these novel stimulants consist of isomers. The distribution of germination stimulation activities on *O. minor* was similar to that on *S. hermonthica* seeds after RP-HPLC separation of maize root exudates, and there were at least 5 active fractions eluting at 8–8.5 min (SL1 isomers), 9–9.5 min (orobanchol, strigol, and sorgomol), 10–11.5 min (SL2 and methoxy-5-deoxystrigol isomers) and 14.5–15 min (5-deoxystrigol). The fraction eluted at 12–13.5 min was found to have a lipophilicity similar to that of orobanchyl acetate, but this SL was not detected in LC–MS/MS analysis (Fig. 3-3). The novel stimulants, SL1, SL2, and methoxy-5-deoxystrigol

isomers afforded the sodium adduct ions at *m/z* 371, 399 and 383 in ESI–MS as shown in Fig. 3-4 and the molecular weight were also confirmed by the molecular ions at *m/z* 376 for SL2, and 360 for methoxy-5-deoxystrigol isomers, while the molecular ion of SL1 was not observed in GC–MS analyses as shown in Fig. 3-5.

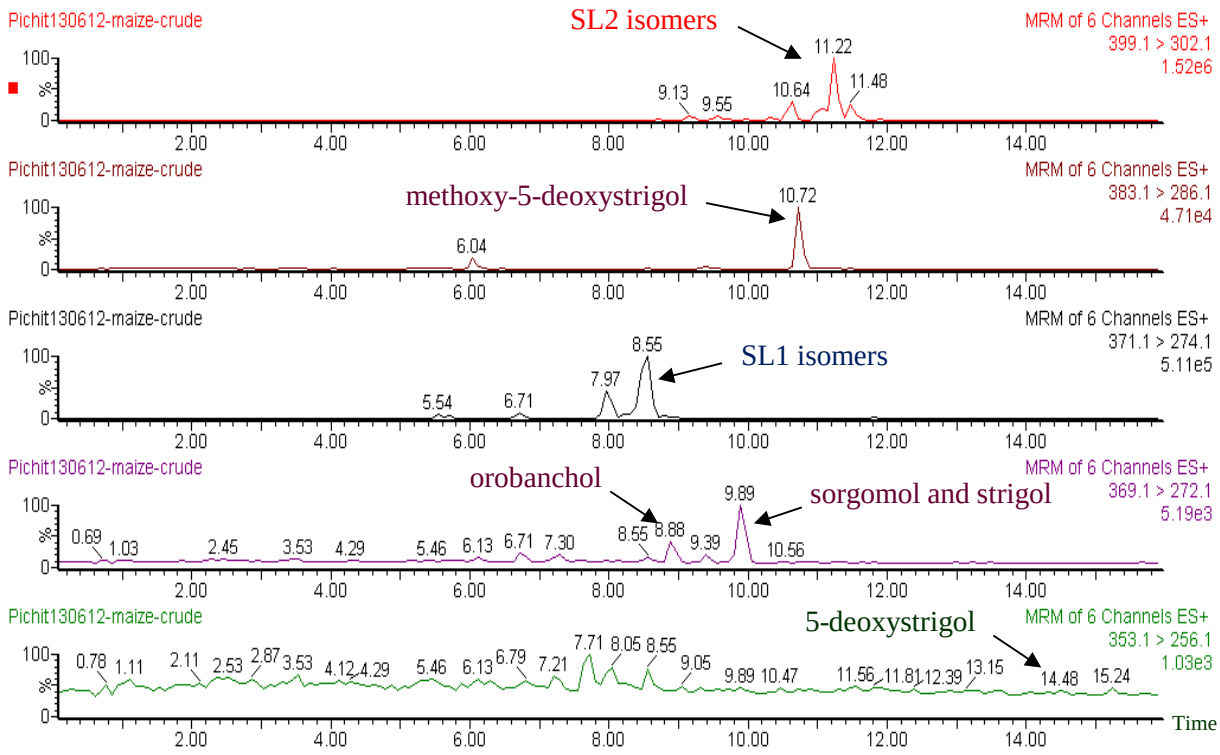


Fig. 3-2. LC–MS/MS (MRM) chromatograms of maize root exudates. The transitions of *m/z* 353 > 256, 369 > 272, 371 > 274, 383 > 286, and 399 > 302 were monitored for 5-deoxystrigol, orobanchol, (sorgomol and strigol), SL1 isomers, methoxy-5-deoxystrigol and SL2 isomers, respectively.

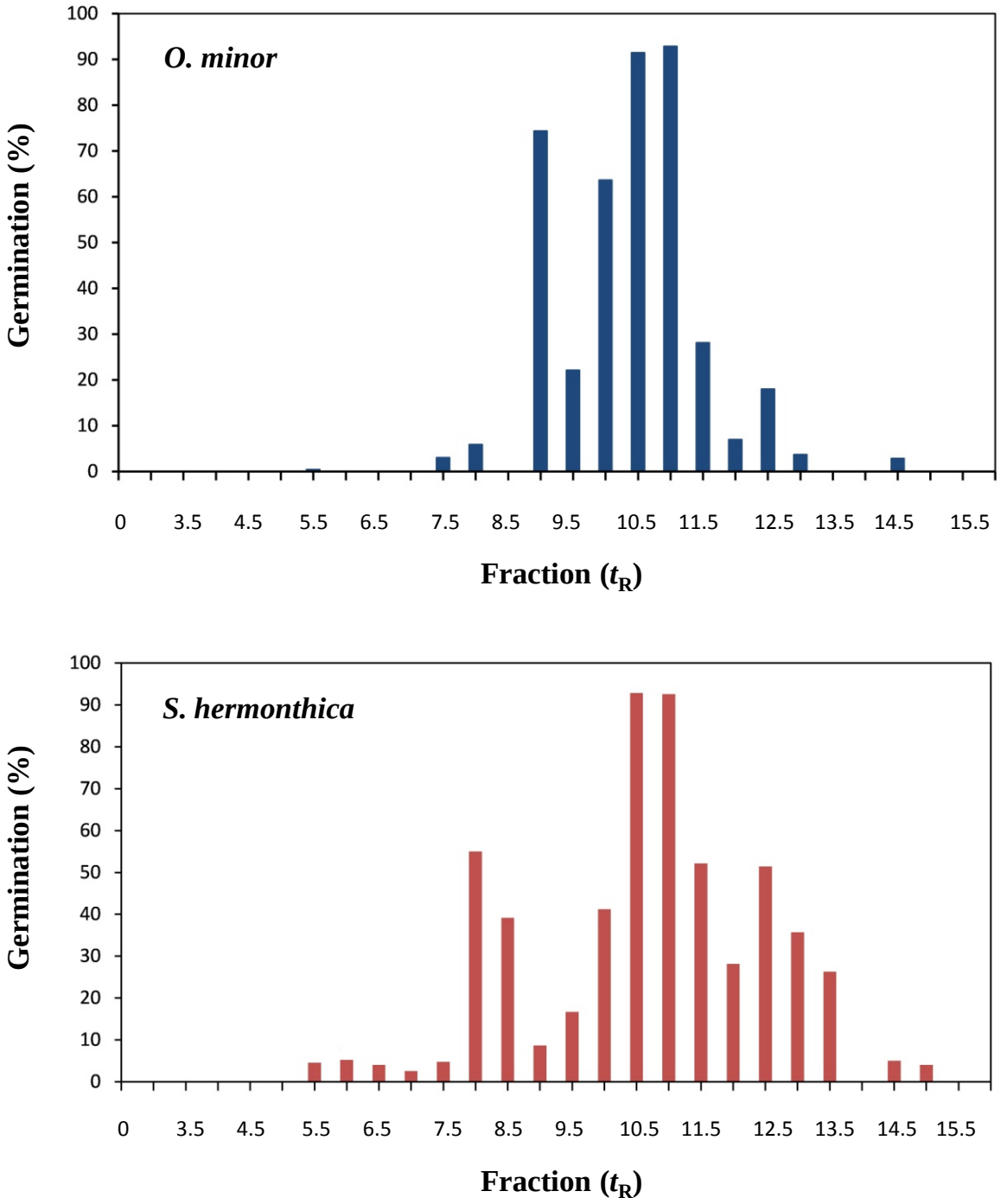


Fig. 3-3. Distribution of germination stimulation activity toward *O. minor* and *S. hermonthica* seeds after RP-HPLC separation of maize root exudates.

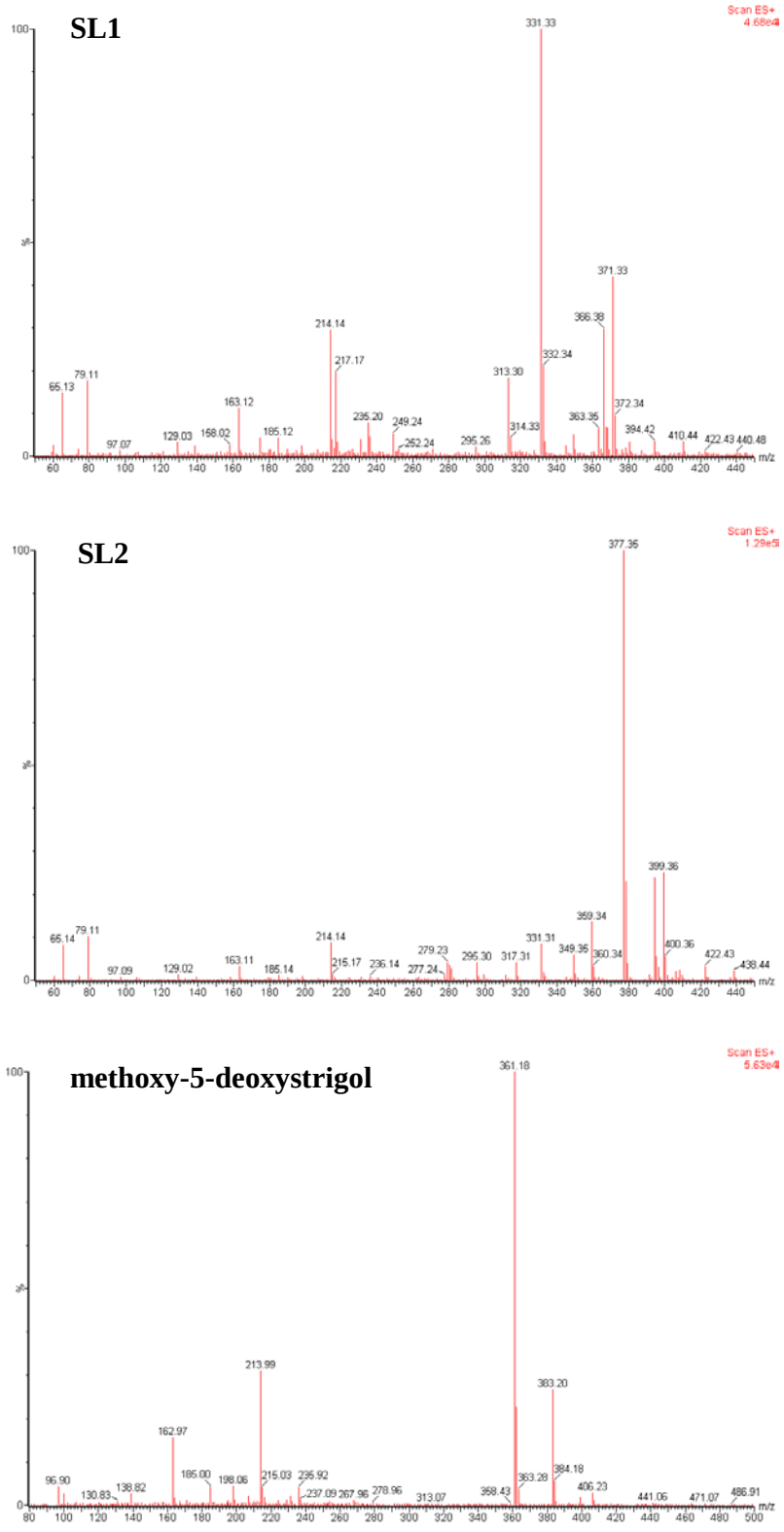


Fig. 3-4. ESI–MS spectra of SL1, SL2, and methoxy-5-deoxystrigol found in maize root exudates.

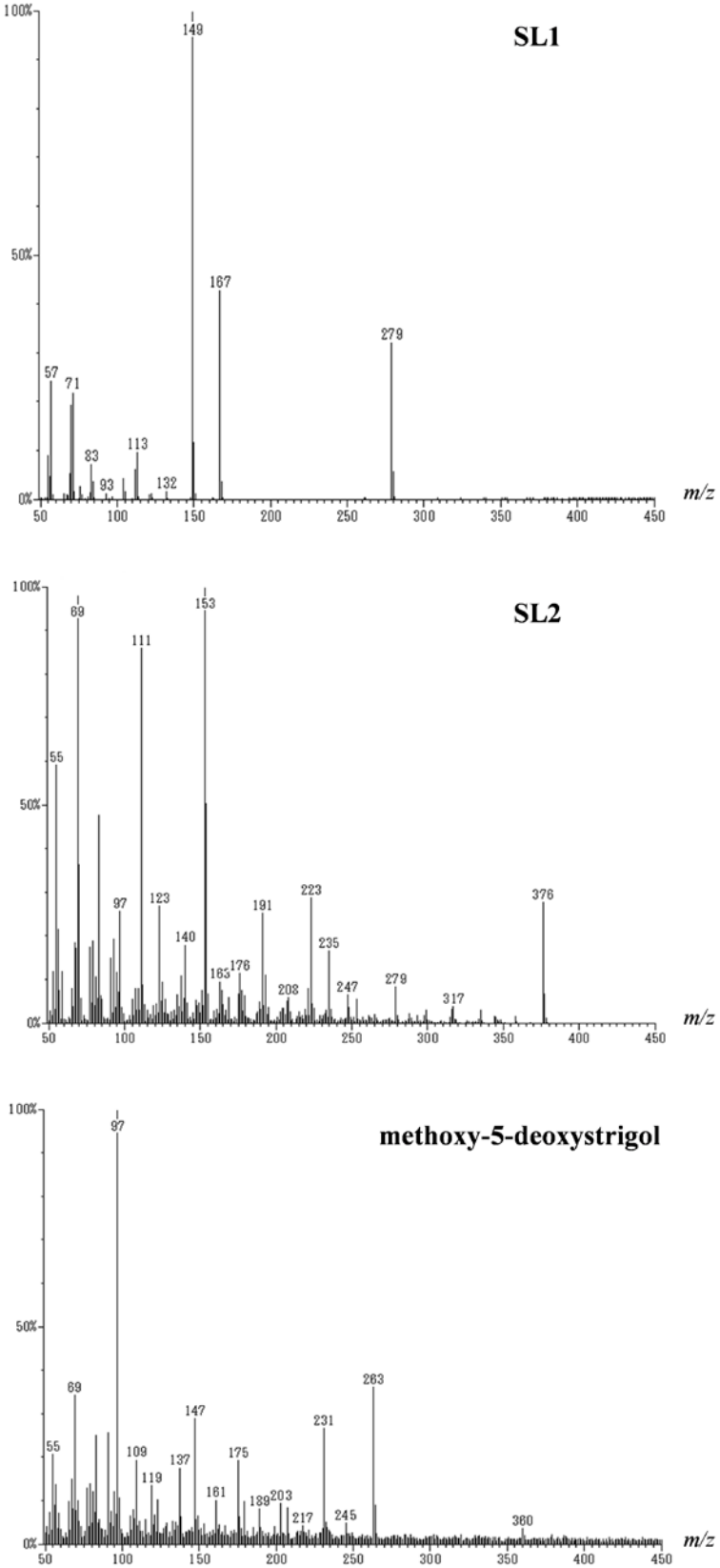


Fig. 3-5. GC–MS spectra of SL1, SL2, and methoxy-5-deoxystrigol found in maize root exudates.

3-2-2. Isolation of a novel germination stimulant from maize root exudates

The crude extract of maize root exudates was subjected to silica gel column chromatography using *n*-hexane–EtOAc gradients as eluting solvent to give 11 fractions. All fractions were analyzed by LC–MS/MS, and were examined for their germination activity on *O. minor* seed as shown in Fig. 3-6.

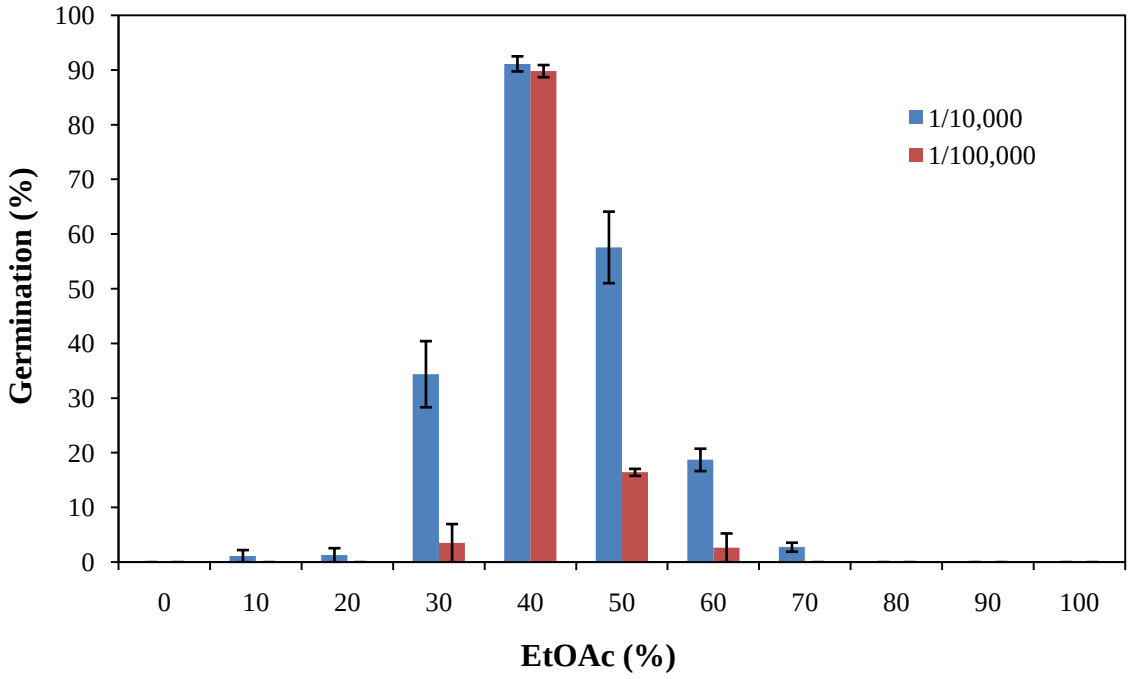


Fig. 3-6. Distribution of germination stimulation activity of maize root exudates toward *O. minor* after silica gel CC. Germination stimulation activity of fractions were examined at 10,000- and 100,000-fold dilutions. Each value is presented as a mean ± SE (n = 3).

The germination stimulants were found to be eluted in the 30–60% EtOAc fractions. By LC–MS/MS analyses (Kisugi et al., 2013), four known SLs were detected in the 50% EtOAc fraction (orobanchol, sorgomol and strigol), the 60% EtOAc fraction (sorgomol and strigol), and the 30% EtOAc fraction (5-deoxystrigol). In addition to these SLs, novel germination stimulants were detected in the 60% EtOAc fraction (SL1 isomers), and the 40% EtOAc fraction (SL2 and methoxy-5-deoxystrigol isomers). The 40% EtOAc fraction induced high germination (~90%) at 10,000- and 100,000-fold dilutions and was found to contain novel germination stimulants, SL2 isomers as major components. This fraction was subjected to silica gel CC and HPLCs to afford novel stimulant **30**.

The molecular formula of novel stimulant **30** was established as $C_{20}H_{24}O_7$ by HR–TOF–MS, ESI–MS and GC–MS analyses. The ^{1}H NMR spectrum of stimulant **30** showed signals for the D ring moiety at δ 1.19 (3H, t, J = 1.5 Hz), 4.88 (1H, quin, J = 1.2 Hz), 5.46 (1H, quin, J = 1.6 Hz), 7.39 (1H, s) ppm. In addition, the absence of typical B-C ring signals corresponding to H-3a, H-4 and H-8b in SLs and the presence of two *trans*-olefinic proton signals at δ 6.89 (1H, d, J = 13.8 Hz) and 7.25 (1H, d, J = 13.8 Hz), methyl signals at δ 1.57, and methyl ester signal at δ 3.28 ppm indicate that this compound contains the A and D rings of typical SLs but lacking the B and C rings. Based on the above spectroscopic data, this compound was assigned to be methyl 9-desmethyl-4-hydroxy-5-oxocarlactone-9-carboxylate (**30**), which may be derived from carlactone (**23**), a

biosynthetic intermediate for SLs as shown in Fig. 3-7. Since the stereochemistry at C-2′ of all natural SLs is an *R*-configuration, (Yoneyama et al., 2013), the stereochemistry at C-11 of compound **30** would also be an *R*-configuration, which is corresponding to position C-2′ of SLs. The quantity of purified compound was enough for ^{1}H NMR measurement but not for ^{13}C and 2D NMR spectroscopy, and therefore further study is needed to confirm the tentative structure.

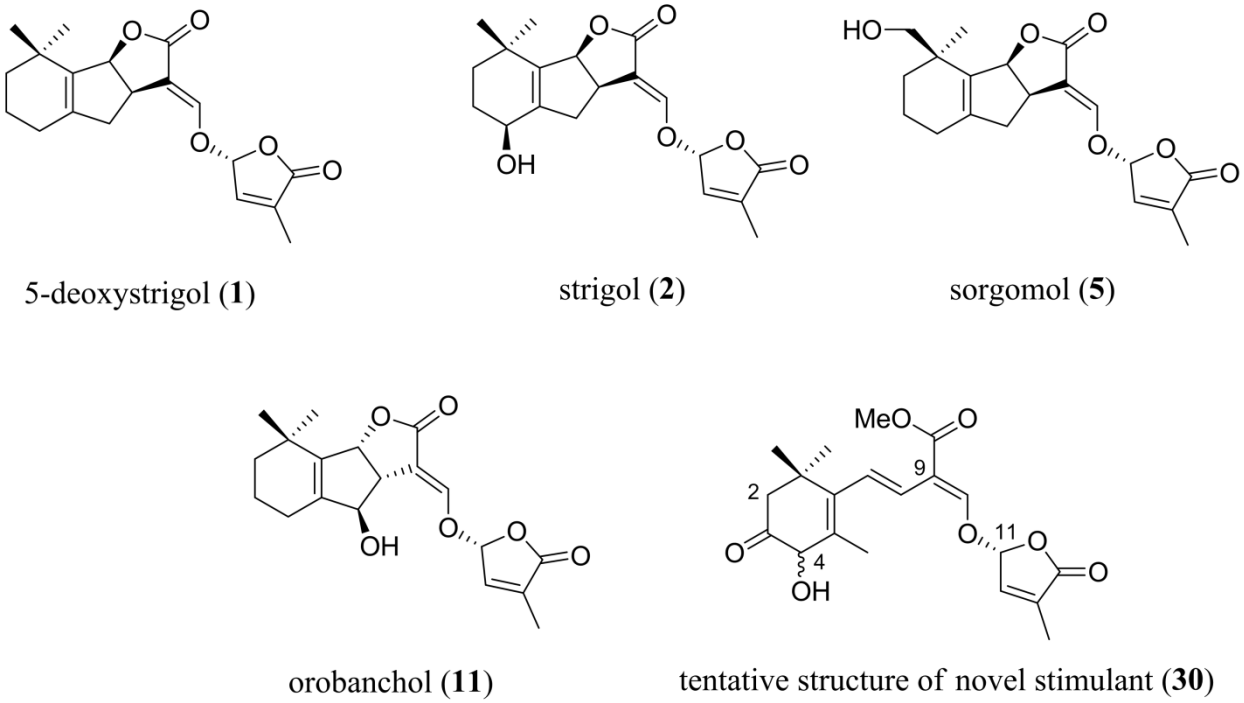


Fig. 3-7. Chemical structures of strigolactones isolated from maize plants; 5-deoxystrigol (**1**), strigol (**2**), sorgomol (**5**), orobanchol (**11**), and novel germination stimulant, SL2 (**30**).

Chapter 4. Conclusion

Among germination stimulants produced by plants, SLs were found to be the most important ones as they have other biological functions in the rhizosphere and also in the plants. Cucumber plants were found to produce and exude mixture of at least 12 germination stimulants including five known SLs, orobanchol (**11**), orobanchyl acetate (**12**), 7-oxoorobanchol (**15**), 7-oxoorobanchyl acetate (**16**), and 4-deoxyorobanchol (**10**), indicating that cucumber plants produce mainly orobanchol-type SLs derived from 4-deoxyorobanchol (**10**) (Chapter 2). By contrast root exudates from maize plants were found to contain at least 11 germination stimulants including four known SLs, strigol (**2**), sorgomol (**5**), 5-deoxystrigol (**1**), and orobanchol (**11**), suggesting that this plant produce both orobanchol- and strigol-type SLs (Chapter 3). In general, SLs have been shown to be widely distributed in the plant kingdom and various SLs could be produced starting from a common precursor, 5-deoxystrigol (**1**) or 4-deoxyorobanchol (**10**) by using biosynthetic enzymes (Xie et al., 2010). 7α- and 7β-Hydroxyorobanchol (**24, 25**) and their acetates (**26, 27**) that are derived from4-deoxyorobanchol (**10**) were purified from cucumber root exudates (Chapter 2). An allylic hydroxylation of 4-deoxyorobanchol (**10**) leads to orobanchol (**11**), and the subsequent oxidation at homoallylic position affords 7-hydroxyorobanchols (**24, 25**). These hydroxyl-SLs are then acetylated to give 7-hydroxyorobanchyl acetates (**26, 27**) as shown in Fig. 4-1. The germination stimulation activities of 7α- and 7β-hydroxyorobanchyl

acetate (**26, 27**) on *O. minor* were comparable to those of orobanchyl acetate (**12**) and 7-oxoorobanchyl acetate (**16**), while germination stimulation activity of the inseparable mixture of 7-hydroxyorobanchols could not be determined due to their instabilities. However, these unstable SLs may be involved in the stimulation of parasite seed germination in the rhizosphere whose chemical and biological characteristics differ profoundly from those of bulk soil. It is intriguing that 7β-hydroxyorobanchyl acetate (**27**) was a highly potent germination stimulant for *P. ramosa* inducing more than 50% germination at 10 pM. This implies that different root parasites may respond to SLs with different sensitivities.

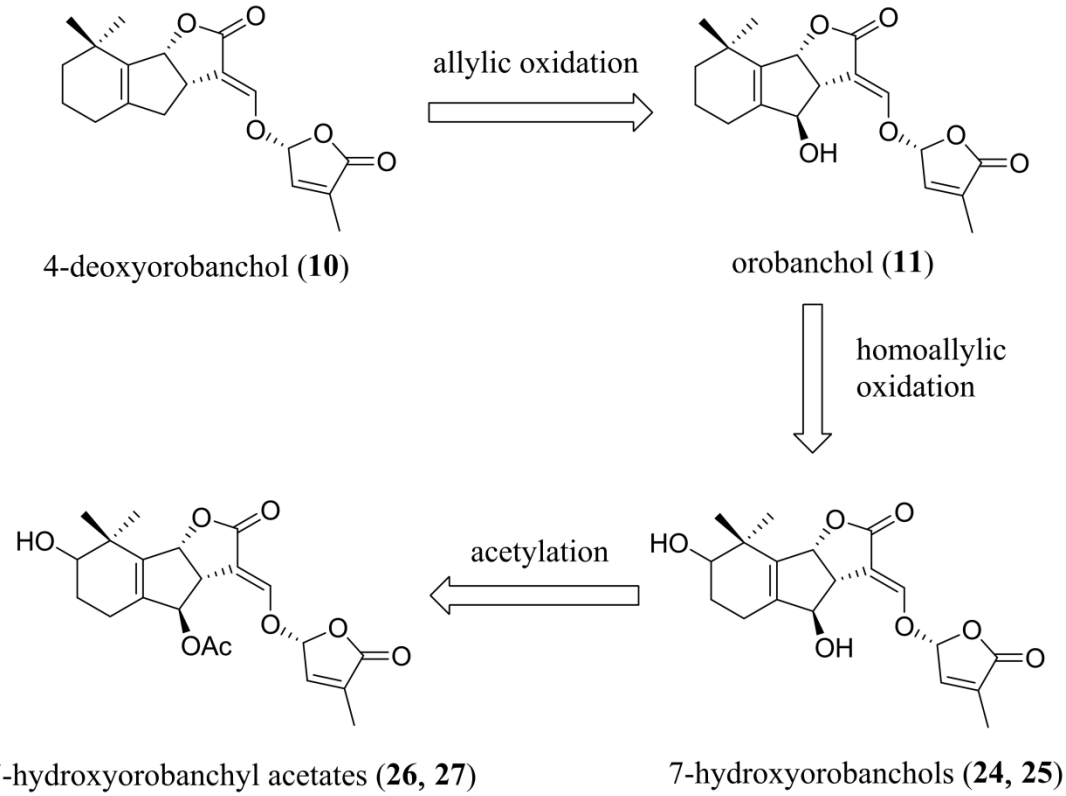


Fig. 4-1. Proposed biosynthesis pathway for 7-hydroxyorobanchols (**24**, **25**) and their acetates (**26, 27**) in cucumber.

In addition to these SLs, novel germination stimulants that are structurally distinct from typical SLs were identified from both cucumber and maize root exudates. These novel stimulants appear to be derived from carlactone, an apocarotenoid biosynthetic intermediate for SLs (Alder et al. 2012).

Very recently, avenaol (**31**) was isolated as a germination stimulant purified from black oat (*Avena strigosa*) root exudates (Kim et al., 2014). Avenaol contains the C-D ring moiety, the common structure for SLs, but lacks the B ring. In my study, cucumber and maize plants were found to produce and exude not only known and new SLs but also novel germination stimulants structurally related to carlactone (Chapters 2 and 3). The deduced chemical structures of these compounds contain the A and the D rings and are similar to carlactone (**23**) rather than typical SLs. The tentative biosynthetic pathway for these germination stimulants is shown in Fig. 4-2.

Since all plant species so far examined have been shown to produce and release mixtures of SLs, it is intriguing to understand if root parasitic weeds, AM fungi, and other beneficial or pathogenic microorganisms need specific mixtures of SLs for their host recognition. Furthermore, the roles of novel type of germination stimulants structurally related to carlactone (**23**) in the chemical communications in the rhizosphere and hormonal regulation of plant growth and development need to be clarified.

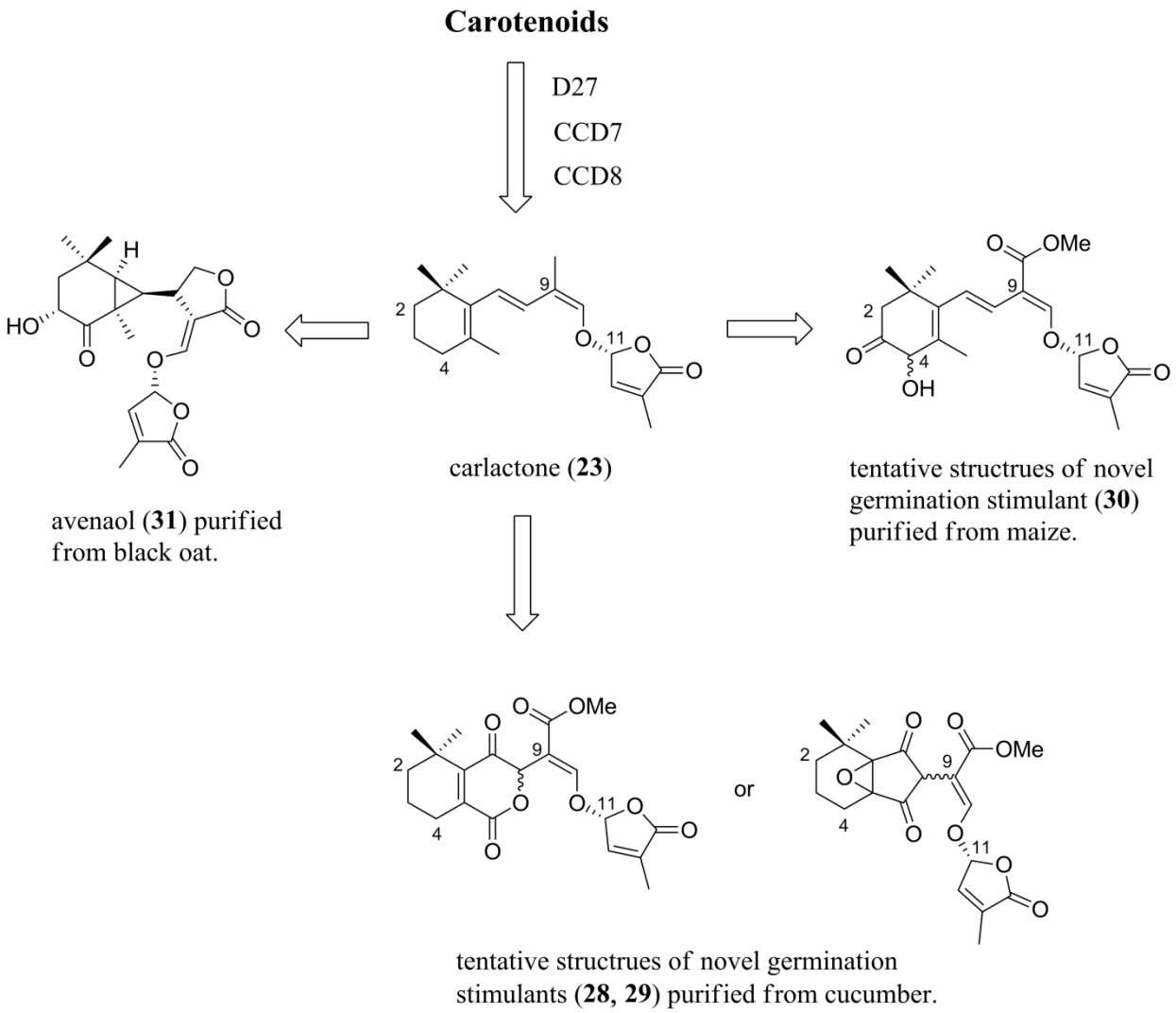


Fig. 4-2. Proposed biosynthesis pathway for novel germination stimulants (**28–31**) produced in black oat, cucumber, and maize.

References

Agusti, J., S. Herold, M. Schwarz, M. Sanchez, K. Ljung, E. A. Dun, P. B. Brewer, C. A. Beveridge, T. Sieberer, E. M. Sehr and T. Greb. 2011. Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. Proc. Natl. Acad. Sci. USA 108: 20242–20247.

Akiyama, K., K. Matsuzaki and H. Hayashi. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435: 824–827.

Alder, A., M. Jamil, M. Marzorati, M. Bruno, M. Vermathen, P. Bigler, S. Ghisla, H. J. Bouwmeester, P. Beyer and S. A. Babili. 2012. The path from β-carotene to carlactone, a strigolactones-plant hormone. Science 335: 1348–1351.

Awad, A. A., D. Sato, D. Kusumoto, H. Kamioka, Y. Takeuchi and K. Yoneyama. 2006. Characterization of strigolactones, germination stimulants for the root parasitic plants *Striga* and *Orobanche*, produced by maize, millet and sorghum. Plant Growth Regul.48: 221–227.

Bouwmeester, H. J., R. Matusova, S. Zhongkui and M. H. Beale. 2003. Secondary metabolite signaling in host-parasitic plant interactions. Curr. Opin. Plant Biol. 6: 358–364.

Bouwmeester, H. J., C. Roux, J. A. Lopez-Raez and G. Bécard. 2007. Rhizosphere communication of plants, parasitic plants and AM fungi. Trends Plant Sci. 12:224–230.

Dube, M. P. and A. Oliver. 2001. *Striga gesnerioides* and its host, cowpea: interaction and methods of control. Can. J. Bot. 79: 1225–1240.

Gomez-Roldan, V., S. Fermas, P. B. Brewer, V. Puech-Pagès, E. A. Dun, J. -P. Pillot, F. Letisse, R. Matusova, S. Danoun, J. -C. Portais, H. J. Bouwmeester, G. Bécard, C. A. Beveridge, C. Rameau and S. F. Rochange. 2008. Strigolactone inhibition of shoot branching. Nature 455: 189–194.

Jamil, M., F. K. Kanampiu, H. Karaya, T. Charnikhova and H. J. Bouwmeester. 2012. *Striga hermonthica* parasitism in maize in response to N and P fertilizers. Field Crop Res. 134: 1–10.

Jeol, D. M., J. C. Steffens and D. E. Matthews. 1995. Germination of weedy root parasites. In: (J. Kigel and G. Galili, eds), Seed development and germination, Marcel Dekker, Inc., New York, pp 567–597.

Jeol D. M. 2000. The long-term approach to parasitic weeds control: manipulation of specific developmental mechanisms of the parasite. Crop Prot. 19: 753–758.

Jeol, D. M., J. Hershenhorn, H. Eizenburg, R. Aly, G. Ejeta, P. J. Rich, J. K. Ransom, J. Sauerborn and D. Rubiales. 2007. Biology and management of weedy root parasites. In: Janick J (ed) Horticultural reviews. Wiley, London, pp 267–349.

Khetkam, P., X. Xie, T. Kisugi, H. I. Kim, K. Yoneyama, K. Uchida, T. Yokota, T. Nomura and K. Yoneyama. 2014. 7α- and 7β-Hydroxyorobanchyl acetate as germination stimulants for root parasitic weeds produced by cucumber. J. Pestic. Sci. 39: 121–126.

Kim, H. I., X. Xie, H. S. Kim, J. C. Chun, K. Yoneyama, T. Nomura, Y. Takeuchi and K. Yoneyama. 2010. Structure–activity relationship of naturally occurring strigolactones in *Orobanche minor* seed germination stimulation. J. Pestic. Sci. 35: 344–347.

Kim, H. I., T. Kisugi, P. Khetkam, X. Xie, K. Yoneyama, K. Uchida, T. Yokota, T. Nomura, C. S. P. McErlean and K. Yoneyama. 2014. Avenaol, a germination stimulant for root parasitic plants from *Avena strigosa*. Phytochemistry 103: 85–88.

Koltai, H. 2011. Strigolactones are regulators of root development. New Phytol. 190: 545–549.

Matusova, R., K. Rani, F. W. Verstappen, M. C. Franssen, M. H. Beale and H. J. Bouwmeester. 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. Plant Physiol. 139: 920–34.

Matuura, H., K. Ohashi, H. Sasako, N. Tagawa, Y. Takano, Y. Ioka, K. Nabeta and T. Yoshihara. 2008. Germination stimulant from root exudates of *Vigna unguiculata*. Plant Growth Regul. 54: 31–36.

Parker, C. and C. R. Riches. 1993. Parasitic weeds of the world: biology and control. CAB International, Wallingford, UK.

Pepperman, A. B. and J. M. Bradow. 1988. Strigol analogs as germination regulators in weed and crop seed. Weed Sci. 36: 719–725.

Pollock, C. B., H. Koltai, Y. Kapulnik, C. Prandi and R. I. Yarden. 2012. Strigolactones: A novel class of phytohormones that inhibit the growth and survival of cancer cells and cancer stem-like enriched mammosphere cells. Breast Cancer Res. Treat. 134: 1041–1055.

Ruyter-Spira, C., W. Kohlen, T. Charnikhova, A. van Zeijl, L. van Bezouwen, N. de Ruijter, C. Cardoso, J. A. Lopez-Raez, R. Matusova, R. Bours, F. Verstappen and H. J. Bouwmeester. 2011. Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in *Arabidopsis*: another belowground role for strigolactones? Plant Physiol. 155: 721–734.

Seto, Y., A. Sado, K. Asami, A. Hanada, M. Umehara, K. Akiyama and S. Yamaguchi. 2014. Carlactone is an endogenous biosynthetic precursor for strigolactones. Proc. Natl. Acad. Sci. USA 111: 1640–1645.

Siame, B. A., Y. Werasuriya, K. Wood, G. Ejeta and L. G. Butler. 1993. Isolation of strigol, a germination stimulant for *Striga asiatica*, from host plants. J Agric. Food. Chem. 41: 1486–1491.

Snowden, K. C., A. J. Simkin, B. J. Jenssen, K. R. Templeton, H. M. Loucas, J. L. Simons, S. Karunairetnam, A. P. Gleave, D. G. Clark and H. J. Klee. 2005. The *Decreased Apical Dominance1/Petunia hybrid CAROTENOID CLEAVAGE DIOXYNEGASE8* gene affects branch production and plays a role in leaf senescence, root growth, and flower development. Plant Cell 17: 746–759.

Tsuchiya, Y., D. Vidaurre, S. Toh, A. Hanada, E. Nambara, Y. Kamiya, S. Yamaguchi and P. McCourt. 2010. A small-molecule screen identifies new functions for the plant hormone strigolactone. Nat. Chem. Biol. 6: 741–749.

Umehara, M., A. Hanada, S. Yoshida, K. Akiyama, T. Arite, N. Takeda-Kamiya, H. Magome, Y. Kamiya, K. Shirasu, K. Yoneyama, J. Kyozuka and S. Yamaguchi. 2008. Inhibition of shoot branching by new terpenoid plant hormones. Nature 455: 195–200.

Xie, X., K. Yoneyama, D. Kusumoto, Y. Yamada, T. Yokota, Y. Takeuchi and K. Yoneyama. 2008. Isolation and identification of alectrol as (+)-orobanchyl acetate, a germination stimulant for root parasitic plants. Phytochemistry 69: 427–431.

Xie, X., K. Yoneyama, J. Kurita, Y. Harada, Y. Yamada, Y. Takeuchi and K. Yoneyama. 2009. 7-Oxoorobanchyl acetate and 7-oxoorobanchol as germination stimulants for root parasitic plants from flax (*Linum usitatissimum*). Biosci. Biotechnol. Biochem. 73: 1367–1370.

Xie, X., K. Yoneyama and K. Yoneyama. 2010. The strigolactones story. Annu. Rev. Phytopathol. 48: 93–117.

Yoneyama, K., X. Xie and K. Yoneyama. 2013. Handbook of natural product, Ranawat K. G. and Merillon J. M. (ed). Springer-Verlag GmbH, Heidelberg, pp. 3583–3604.

Zwanenburg, B., A. S. Mwakaboko, A. Reizelman, G. Anilkumar and D. Sethumadhavan. 2009. Structure and function of natural and synthetic signaling molecules in parasitic weed germination. Pest Manag. Sci. 65: 478–491.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Prof. Dr. Koichi Yoneyama, for his inspiration, guidance, valuable knowledge, kindness, helpful supervision and hearty encouragement throughout my study and made me succeed in writing this dissertation.

Sincere gratitude is expressed to co-supervisors, Assist. Prof. Dr. Xiaonan Xie for his kind guidance, assistance and helpful advice, Prof. Dr. Masahiro Natsume, Tokyo University of Agriculture and Technology, and Prof. Dr. Yoshihito Suzuki, Ibaraki University for variable suggestions and comments.

I am grateful to Prof. Dr. Apichart Suksamrarn, Ramkhamhaeng University, Thailand, Prof. Dr. Yasutomo Takeuchi, Teikyo University, Assoc. Prof. Dr. Takahito Nomura, Dr. Kaori Yoneyama, Dr. Takaya Kisugi and Dr. Sureeporn Homvisasevongsa, Huachiew Chalermprakiet University, Thailand for kind and helpful advice.

I am indebted to Prof. Dr. Kenichi Uchida, Teikyo University for recording the nuclear magnetic resonance spectra. I am grateful to Prof. Dr. Takao Yokota, Teikyo University for recording the high resolution mass spectra.

I am also grateful to Mr. Hyun Il Kim, Mr. Chairat Burana, the Master, the Senior Project students and the staff of the Center for Bioscience Research and Education, Utsunomiya University for their encouragement and kind assistance.

I would like to express my special thank to my lovely family, Miss Phorntip Sae-lim and her family for hearty encouragement.

Finally, this work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry and by the special grant for UU-COE from Utsunomiya University. Financial support from Japanese Government (Monbukagakusho: MEXT) Scholarship.