JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of Strigolactones, Germination Stimulants for *Striga* and *Orobanche*, by High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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A simple and rapid analytical method for strigolactones, germination stimulants for the root parasitic weeds witchweed (*Striga* spp.) and broomrape (*Orobanche* spp.), has been developed using high-performance liquid chromatography connected to tandem mass spectrometry (LC/MS/MS). The natural strigolactones (strigol, sorgolactone, orobanchol, and alectrol) were clearly separated and identified by LC/MS/MS. As low as 0.1 pg/μL of strigol and 0.5 pg/μL of sorgolactone could be quantified, whereas 1 pg/μL was needed for the quantification of orobanchol (S/N > 10). Using this method, it was found that red clover produces orobanchol and alectrol but not strigol. The roots of red clover seedlings were found to produce 13, 70, 58, and 65 pg of orobanchol/plant 1, 2, 3, and 4 weeks after germination, respectively.

KEYWORDS: LC/MS/MS; strigol; orobanchol; sorgolactone; parasitic weeds; germination stimulants; *Striga* spp.; *Orobanche* spp.

INTRODUCTION

Witchweed (*Striga* spp.) and broomrape (*Orobanche* spp.) are two of the most devastating root parasitic weeds, causing severe damage to agricultural production (*1*). Seeds of these parasites germinate only when they perceive chemical signals, that is, germination stimulants produced by and released from the roots of host and nonhost plants. To date, four different stimulants belonging to a chemical class referred to as strigolactones have been isolated from root exudates of host and nonhost plants (**Figure 1**). These are strigol (from cotton) (*2*, *3*), sorgolactone (from sorghum) (*4*), alectrol (from cowpea) (*5*), and orobanchol (from red clover) (*6*). These stimulants, even at concentrations lower than 10^{-9} M, strongly elicit *Striga* and *Orobanche* seed germination and, thus, are considered to play a pivotal role in host recognition by these root parasites. In some cases, hosts that are hardly affected by the parasites have been reported to produce only minute amounts of germination stimulants (*7*).

So far, the relative amounts of strigolactones in root exudates have been estimated on the basis of their germination-inducing activity in bioassays, because germination tests using *Striga* and *Orobanche* seeds are highly sensitive and specific to these stimulants (*8*). However, root exudates may also contain germination inhibitors, and therefore the amounts of germination stimulants may sometimes be underestimated. In addition, some host plants such as red clover for *Orobanche* and sorghum for *Striga* have been reported to produce more than one stimulant (*6*, *9*). Therefore, these host plants may produce individual stimulants at different levels under different growth conditions and/or at different growth stages.

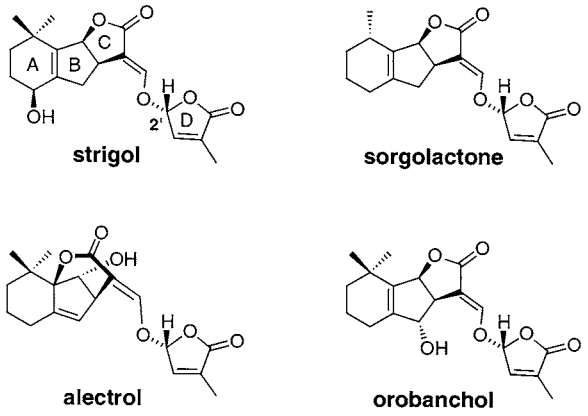

Figure 1. Chemical structures of strigolactones.

Environmental factors including temperature, photoperiod, nutrient availability, soil type, and moisture may directly and indirectly affect the production of germination stimulants. For example, Weerasuriya et al. have shown that much greater

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10.1021/jf025997z CCC: $25.00
Published on Web 01/18/2003

stimulant activity was detected in plants grown under a short-day regime (*10*). In the case of red clover, when grown hydroponically, nutrients in the growth medium were found to affect stimulant production, where nitrate and urea promoted but ammonium and phosphorus inhibited stimulant production (*11*). Indirect effects of nutrients on seed germination could be eliminated by extracting root exudates or culture media with organic solvents.

Strigolactones could be analyzed by gas chromatography/mass spectrometry (GC/MS) after time-consuming and sophisticated purification (*6*), but partial decomposition of these rather unstable strigolactones seems to occur during purification. For this reason, strigolactones in the root exudates should be characterized prior to the addition of internal standards. Therefore, the development of a rapid and simple analytical method for strigolactones is essential for the exploration of the possible distribution of strigolactones in the plant kingdom and also for clarifying their role in host recognition mechanisms of root parasites.

Recently, high-performance liquid chromatography connected to tandem mass spectrometry (LC/MS/MS) has extensively been used to analyze thermolabile and unstable compounds including peptides and proteins. In particular, a selective detection by means of multiple reaction monitoring (MRM) can be applied for the analysis of trace amounts of compounds in crude samples (*12*). Therefore, LC/MS/MS was also expected to provide a useful analytical method for strigolactones in root exudates. However, so far there has been no report of LC/MS/MS analyses of strigolactones.

In the present paper, the development of a rapid and simple analytical method for strigolactones by LC/MS/MS and the identification and quantification of strigolactones in root exudates of red clover are described.

MATERIALS AND METHODS

Chemicals. (+)-Strigol and (+)-orobanchol were generously provided by Emeritus Prof. Kenji Mori (The University of Tokyo). (±)-Sorgolactone was prepared as described in the literature (*13*). Alectrol was isolated from red clover root exudates (*6*). The amounts of alectrol were not enough to be weighed accurately. Other chemicals of analytical grade and HPLC solvents were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan).

Source of Seeds. Red clover (*Trifolium pratense* L. cv. Hamidori) seeds were purchased from local markets. Clover broomrape (*Orobanche minor* Sm.) seeds were collected from mature plants that parasitized on red clovers grown either in the Watarase basin (1998 and 1999) or in an experimental field of Utsunomiya University (2000 and 2001).

Collection of Root Exudates from Red Clover. Red clover seeds were surface-sterilized in 70% ethanol for 2 min and then in 1% sodium hypochlorite for 10 min. After a thorough rinse with sterilized distilled water, they were germinated on a moistened filter paper in Petri dishes for 3 days at 25 °C in the dark. Seedlings ($n = 400$) were transferred to a strainer (28 × 23 × 9 cm, W × L × H) lined with a sheet of gauze moistened by placing it in a slightly larger container (28.5 × 23.5 × 11 cm) containing 2.5 L of sterilized tap water as the culture medium. Sterilized tap water was used as the cluture medium for red clover, because tap water was found to be a good medium for the production of germination stimulant by red clover (*6*). Four of these strainers in the containers were transferred to a growth room at 25 °C with continuous fluorescent illumination (~60 μmol m^{-2} s^{-1}). Sterilized tap water was applied daily to maintain the water level. The tap water medium was replaced with 2.5 L of fresh sterilized tap water weekly. The strainers and containers were thoroughly washed with sterilized tap water when the medium was collected. Approximately 12 L of the tap water medium (plus washing) collected weekly was extracted with ethyl acetate. The ethyl acetate extracts were washed with 0.5 M phosphate buffer (pH 8.5), dried over anhydrous magnesium sulfate, and concentrated in vacuo.

Germination Assays. Germination assays using *O. minor* seeds were conducted as reported previously (*11*), except that the temperature for conditioning and germination was set to 23 °C.

HPLC. HPLC separation was done with a JASCO U980 HPLC instrument (JASCO, Tokyo, Japan) equipped with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 mm, 5 μm, Kanto Chemicals Co. Ltd., Tokyo, Japan). The mobile phase was 55% methanol in water and switched to 100% methanol 40 min after injection. The column was then washed with 100% methanol for 20 min to elute all injected materials. The flow rate was 0.2 mL/min. Column temperature was set to 40 °C.

Mass Spectrometry. Mass spectrometry was performed on a Quattro LC mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray source. Drying gas as well as nebulizing gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to ~100 L/h and the desolvation gas flow to 500 L/h. The interface temperature was set to 400 °C and the source temperature to 150 °C. The capillary and cone voltages were adjusted to each molecule and to the ionization mode (positive and negative). MS/MS experiments were done using argon as collision gas, and the collision energy was optimized for each compound. The collision gas pressure was 1.5 × 10^{-3} mbar. Data acquisition and analysis were performed using MassLynx software (ver. 3.2) running under Windows NT (ver. 4.0) on a Pentium PC.

RESULTS AND DISCUSSION

Electrospray Mass Spectrometry of Strigolactones. Figure 2 shows the full-scan positive mass spectrum of strigol (1 μM in 55% methanol, delivered with an infusion pump at 5 μL/min) for which the capillary and the cone voltages were set to 3.5 kV and 40 V, respectively. No significant ions were observed in the negative mass spectrum (data not shown). The sodium adduct ion, $[M + Na]^+$, of *m*/*z* 369 was the most abundant, whereas the intensity of the protonated molecule, $[M + H]^+$, of *m*/*z* 347 was quite weak. The potassium adduct ion, $[M + K]^+$, of *m*/*z* 385 was also detected. Addition of acetic acid to the solution (up to 0.25%, v/v) did not increase the intensity of the protonated molecule ion, but the intensity of the sodium adduct ion became slightly unstable in a long run. The only clear fragment ion at *m*/*z* 272 can be explained by cleavage of the vinyl ether bond between the C and D rings (**Figure 1**). Similar fragmentations were observed for electron impact ionization (EI) and chemical ionization (CI) mass spectra of strigol and its derivatives (*4*−*6*, *9*). In the full-scan positive mass spectra of orobanchol and sorgolactone, corresponding sodium adduct ions were also most abundant (data not shown). Because the sodium adduct ions of these strigolactones were observed for effluents from ODS-HPLC, these were selected as parent or precursor ions for collision-induced degradation (CID).

The CID spectrum of strigol $[M + Na]^+$ is shown in **Figure 3**. The most abundant ion in this product ion spectrum was at *m*/*z* 272 as a result of neutral loss of the D-ring moiety. The optimal collision energy was found to be 19 eV for strigol. Orobanchol and sorgolactone also showed similar product ion spectra where ions at *m*/*z* 272 and 242 were most abundant, respectively. The optimal collision energies for orobanchol and sorgolactone were 16 and 15 eV, respectively.

Analysis of Strigolactones with the Multiple Reaction Monitoring Method. For MRM, also called selected reaction monitoring (SRM), transition *m*/*z* 369 > 272 was selected for strigol and orobanchol and transition *m*/*z* 339 > 242 for sorgolactone. These two transitions were monitored with two channels. Although optimal collision energies were different for

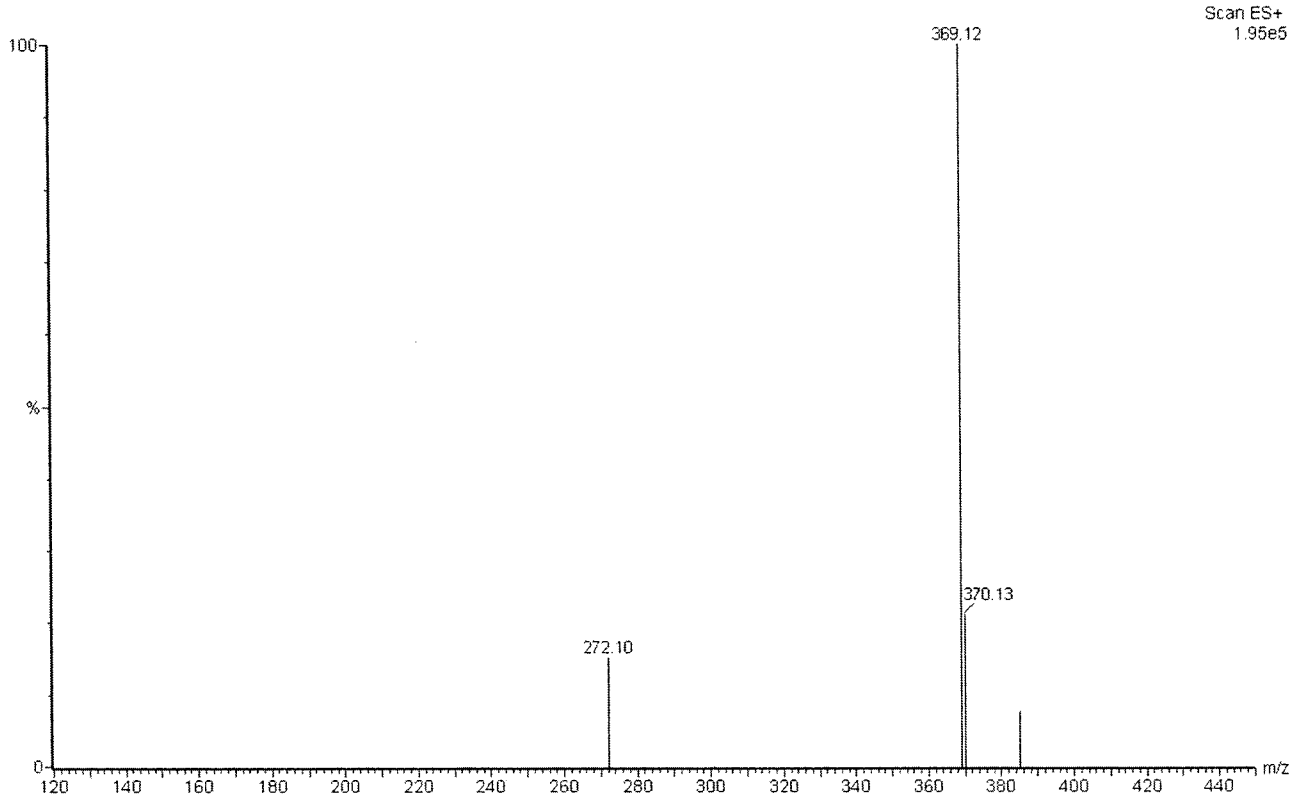

Figure 2. Electrospray-positive ion mass spectrum of authentic strigol.

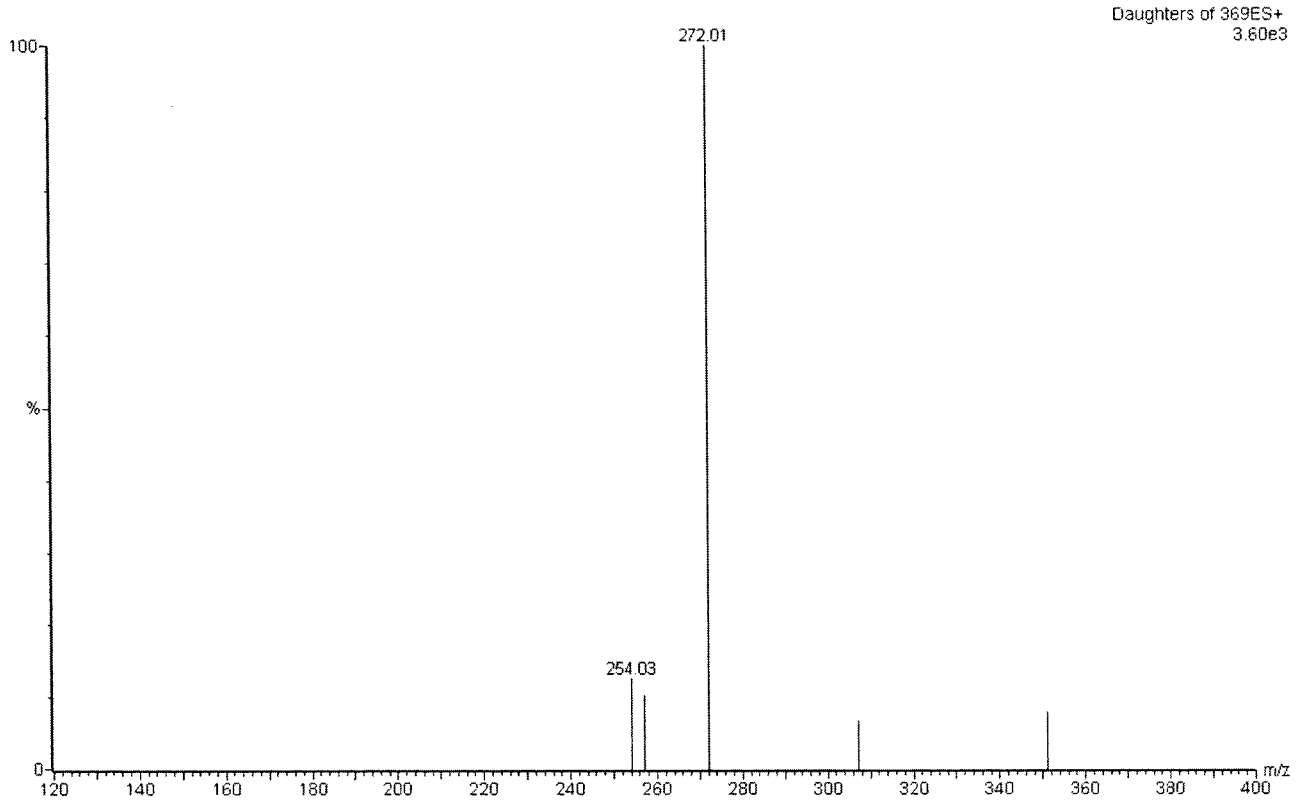

Figure 3. Daughter ion spectrum of strigol $[M + Na]^+$.

strigol and orobanchol, the collision energy for both was set at 16 eV, which is an optimal energy for orobanchol but not high enough for strigol. This is because these two stimulants had similar retentions on the ODS-HPLC and the signal intensity of orobanchol was found to decrease at collision energies higher or lower than 16 eV. By contrast, for strigol, the reduction of signal intensity caused by lowering the collision energy from 19 to 16 eV was almost negligible. It is possible to detect orobanchol and strigol with different channels when the collision energy is set to each optimal value, but the sensitivity may decrease with the reduction in the dwell time due to the increase in the number of channels. The dwell time for the two-channel

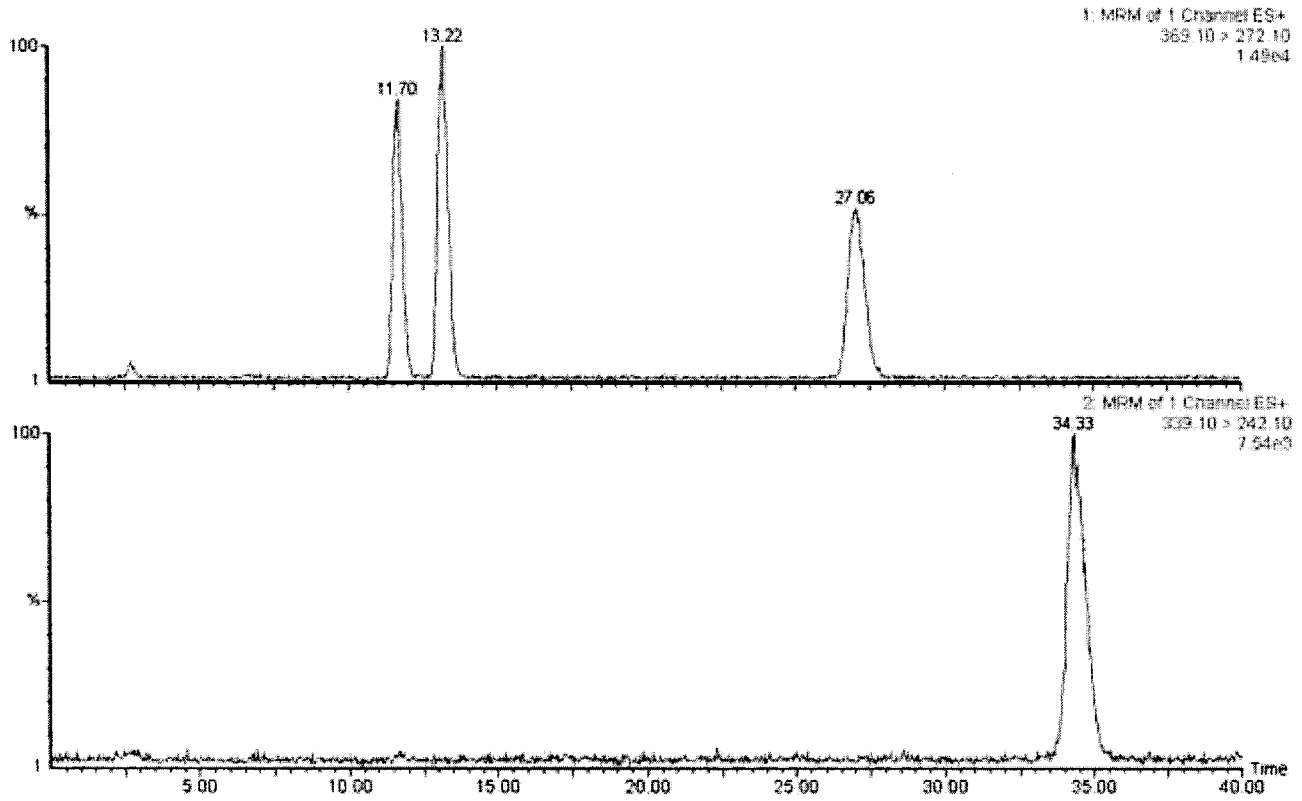

Figure 4. Two-channel MRM chromatogram of a mixture of orobanchol, strigol, alectrol, and sorgolactone.

MRM was set to 0.8 s. These analytical conditions, however, were not optimized for alectrol due to the scarcity of alectrol isolated from red clover root exudates.

A mixture of authentic orobanchol (346 pg), strigol (57.8 pg), alectrol, and sorgolactone (158 pg) in 10 μL of 55% methanol was analyzed by using the two-channel MRM as shown in **Figure 4**. These four strigolactones were clearly separated with retention times (t_R) of 11.7, 13.2, 27.1, and 34.3 min for orobanchol, strigol, alectrol, and sorgolactone, respectively. With electrospray ionization, strigol and sorgolactone showed intense peaks in the MRM chromatogram. The peak intensity of orobanchol was rather weak compared to that of strigol and sorgolactone, and an ~10-fold higher concentration of orobanchol was required to achieve a peak with intensity comparable to that of strigol. For strigol and sorgolactone, detection (S/N > 3) and quantification (S/N > 10) limits were approximately 0.05 and 0.1 pg/μL and 0.1 and 0.5 pg/μL, respectively.

Analysis of Strigolactones in Red Clover Root Exudates. Red clover roots have been shown to produce at least three different germination stimulants, that is, orobanchol, alectrol, and an unknown compound (*6*). Crude ethyl acetate extracts of red clover root exudates were analyzed by using the LC/MS/MS method developed in this study. The ethyl acetate extracts, each corresponding to ~12 L of root exudates, were dissolved in 50−100 μL of 55% methanol and filtered through spin columns (Ultra-Free MC, 0.45 μm pore size, Millipore), and 10 μL aliquots were injected to HPLC.

Figure 5 shows the two-channel MRM chromatogram of red clover root exudates collected 3 weeks after germination. Although there were several peaks in the transition *m*/*z* 339 > 242, two peaks were conspicuous at 30−40 min. However, none of them may be sorgolactone, as we could not detect germination stimulative activity for these fractions as described in a previous paper (*6*). To confirm stimulatory activity, we need to collect larger amounts of red clover root exudates, because some germination inhibitors may not be separated from sorgolactone under the analytical conditions used in the present study.

In the transition *m*/*z* 369 > 272, two intense peaks were detected. According to our previous results (*6*), the first (t_R = 11.2 min) was assigned to orobanchol and the second (t_R = 25.4 min) to alectrol. To confirm these assignments, authentic strigolactones were added to the crude extract. A significant increase in the intensity of the first peak was observed on the addition of authentic orobanchol, indicating that the first peak was orobanchol. Similarly, the addition of pure alectrol that had been isolated from red clover root exudates (*6*) enhanced the second peak, and therefore the second peak was alectrol. By contrast, the addition of authentic strigol did not lead to a similar effect either with the first peak or with the second one (data not shown). Slight reductions in the orobanchol and alectrol retentions on the ODS column compared to that in **Figure 4** may be due to the effects of matrix in the crude extracts.

Figure 6 presents the two-channel MRM chromatogram of red clover root exudates collected 4 weeks after germination. During the third and fourth weeks after germination, red clover roots appeared to produce orobanchol and alectrol in a similar ratio, because the relative peak heights of orobanchol and alectrol were very similar in **Figures 5** and **6**. Unfortunately, at the moment, the amounts of alectrol cannot be calculated, as the amounts of alectrol isolated from red clover root exudates were too small to weigh accurately and its synthetic standard is not yet available. Nevertheless, it was confirmed that red clover produces orobanchol and alectrol but not strigol. Furthermore, the minor unknown stimulant(s) produced by red clover roots does not seem to be a strigol isomer (*6*), because all strigol isomers should have been detected in the transition *m*/*z* 369 > 272 with the LC/MS/MS method.

Quantification of Orobanchol. Orobanchol in the red clover root exudates was quantified using the method established in

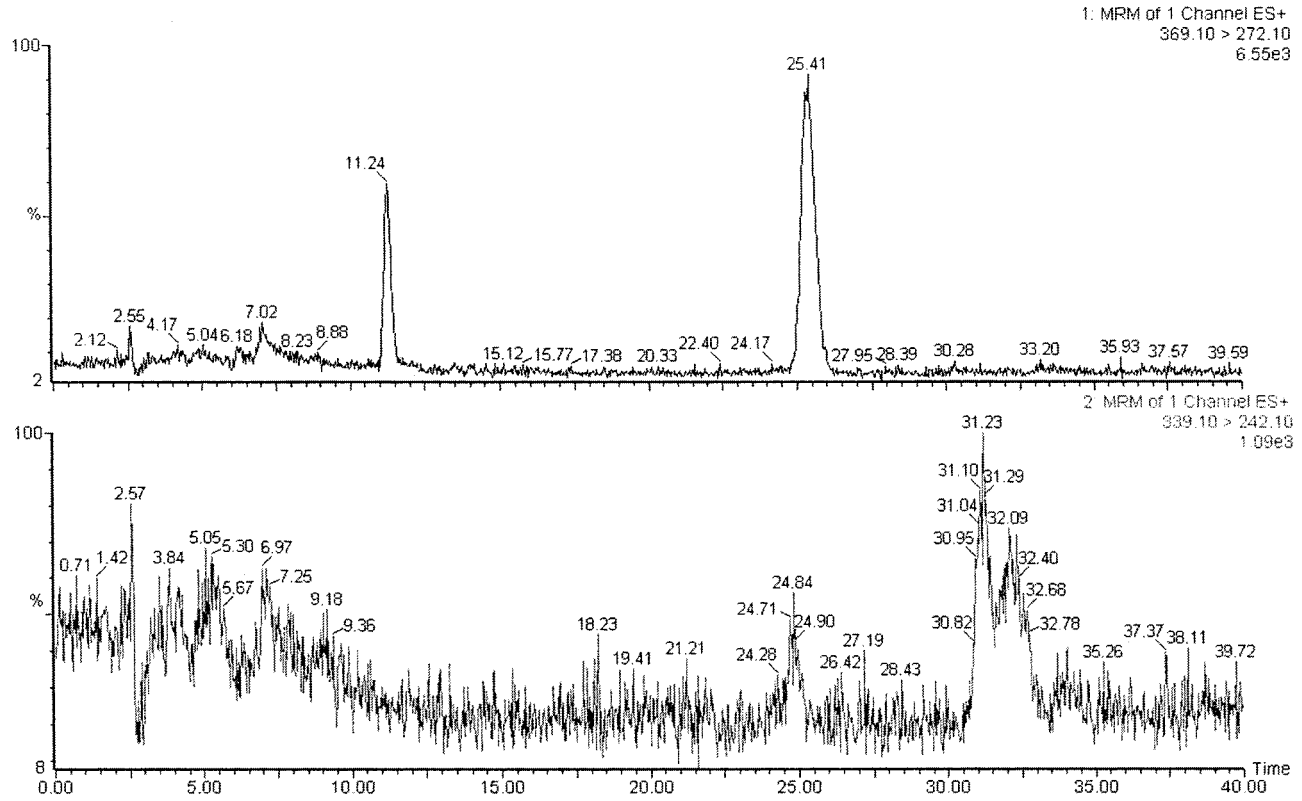

Figure 5. Two-channel MRM chromatogram of red clover root exudates collected 3 weeks after germination.

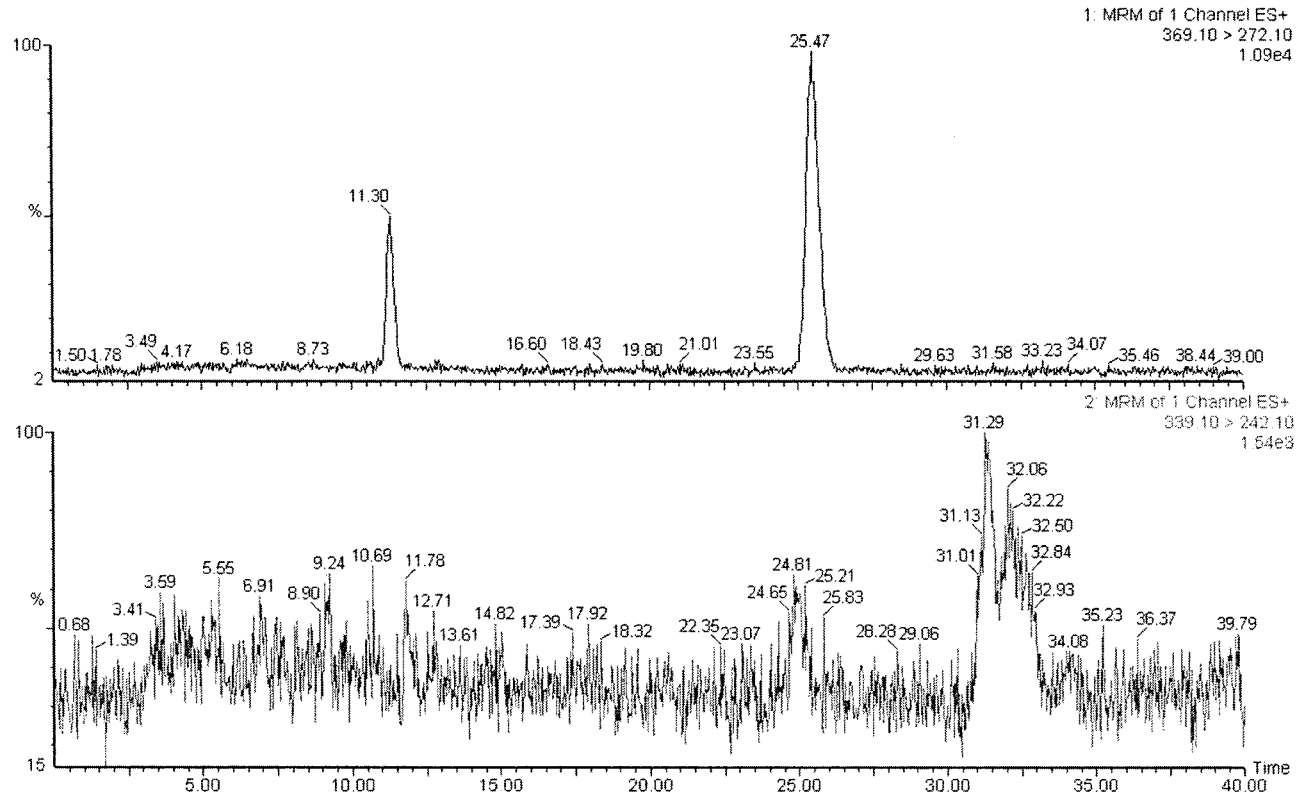

Figure 6. Two-channel MRM chromatogram of red clover root exudates collected 4 weeks after germination.

this study. For this, peak areas or heights may be directly used if ionization efficiency of orobanchol or other strigolactones is not affected by matrix in the crude extract. However, observed increases in peak areas on the addition of known amounts of orobanchol were significantly lower than those expected from the peak areas of the standard strigolactone solution shown in **Figure 4**. For example, a standard solution of 100 pg of orobanchol in 10 μL of 55% methanol gave an ~350-unit area, but an increase of only 80 units was observed when the same amounts of orobanchol were added to the crude extracts of root

Table 1. Orobanchol Production by Red Clover Seedlings Grown Hydroponically[a]

sampling period	total fresh wt (mg/plant)	orobanchol production (pg/plant)	orobanchol production (pg/mg of roots)
first week	32.1 ± 1.0	13	2.6
second week	36.7 ± 1.1	70	11.4
third week	43.9 ± 1.0	58	6.1
fourth week	49.1 ± 1.8	65	6.3

[a] Fresh weight of red clover plants was measured when the root exudates were collected. Values are means of 20 seedlings ± SE ($n = 20$).

exudates collected 3 weeks after germination. Consequently, for quantification of strigolactones in crude extracts, the estimation using a calibration curve generated on the basis of the peak areas of different concentrations of standard is not applicable. Therefore, we analyzed the crude extracts fortified with known amounts of orobanchol. For this, an aliquot of the filtered 55% methanol sample solutions was diluted with a volume of either pure 55% methanol or 55% methanol containing known amounts of orobanchol, so that the solutions thus prepared contained the same amounts of orobanchol, alectrol, and the matrix originally presented in the samples. In this case, areas of alectrol, which should remain constant for both of the solutions, could be used as an internal standard to minimize the errors.

It was thus estimated that the roots of red clover seedlings produced 13, 70, 58, and 65 pg of orobanchol/plant 1, 2, 3, and 4 weeks after germination, respectively (**Table 1**). Of course, these amounts are related not only to the production but also to the degradation of orobanchol. During the 4 weeks after germination, red clover seedlings grew well, and their total fresh weight increased almost linearly. However, further growth in sterilized tap water seemed to be difficult, as most seedlings became chlorotic 4 weeks after germination. The production of orobanchol and alectrol was already observed in the root exudates collected 1 week after germination, and it reached a maximum in the root exudates collected 2 weeks after germination. Then, the stimulant production seemed to decline and stayed at a constant level for the third and fourth weeks after germination. These results indicate that red clover seedlings produce larger amounts of germination stimulants at their early growth stages. Alternatively, actively growing roots may be the major source of germination stimulants.

We have developed a simple and rapid analytical method for strigolactones by LC/MS/MS. Although this analytical method is highly sensitive, strigolactones with unknown structures cannot be detected. In addition, one should keep in mind that peaks observed in the MRM chromatograms are not necessarily strigolactones; they may also be some other compounds that happen to have similar fragmentations under the same analytical conditions. Therefore, it is important to confirm that germination stimulative activity is associated with the peaks observed. This is the main reason we used root exudates of red clover, because we have already examined the germination stimulants produced by this plant.

Natural strigolactones characterized to date contain the D-ring moiety as a common structure and on collision with argon their $[M + Na]^+$ ions lose this moiety as a neutral fragment, resulting in the formation of $[M + Na - 97]^+$ ions. Therefore, if all strigolactones have the same D-ring moiety, novel and known strigolactones should be detected with the neutral-loss (Nloss) mode in MS/MS where the scan functions of the two MS detectors are synchronized with a 97 Da difference corresponding to the loss of the D-ring moiety. Unfortunately, this Nloss method was found to be 500-fold less sensitive than the MRM method described above when standard solutions of strigolactones were analyzed for comparison.

Another strategy is to set the *m*/*z* of precursor and product ions at those of possible strigolactone structures for the MRM analysis. For example, we observed a peak in transition *m*/*z* 367 > 270 in the MRM chromatogram of tomato root exudates, which may indicate the presence of a novel strigolactone that lacks two hydrogens, presumably dehydro-orobanchol (data not shown).

Conclusion. The LC/MS/MS described here enables direct identification and quantification of known strigolactones in the root exudates of various plants and can be used for screening resistant cultivars for low stimulant production or for trap crops for high stimulant production. In addition, this method may clarify if particular resistances to the root parasites depend on stimulant production. Furthermore, the direct quantification of stimulants may help in the detection of inhibitors. The determination and quantification of strigolactones in root exudates of various other host and nonhost plants and their cultivars are in progress.

ACKNOWLEDGMENT

We acknowledge Professor Emeritus Kenji Mori (The University of Tokyo) for his generous gifts of (+)-strigol and (+)-orobanchol. We thank Dr. D. M. Joel (ARO, Israel) for critical reading of the manuscript.

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Received for review October 2, 2002. Revised manuscript received December 11, 2002. Accepted December 13, 2002. Part of the study was supported by a Grant-in-Aid for Scientific Research (B12460049) from the Japanese Society for the Promotion of Science (JSPS) and the Cooperative Research Program of ALRC, Tottori University.

JF025997Z