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A hemiterpene glucoside as a probing deterrent of the bean aphid, *Megoura crassicauda*, from a non-host vetch, *Vicia hirsuta*

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Abstract

The bean aphid, *Megoura crassicauda* Mordvilko, feeds selectively on plants belonging to the genus *Vicia* (Fabaceae). However, it never infests the tiny vetch, *V. hirsuta* (L.) Gray. The aphid appeared to discriminate between host and non-host plants by tasting specific chemicals during penetration of its stylet into the plant tissues. The aphid, after being stimulated by specific probing stimulants, deposited characteristic proteinous stylet sheaths through a parafilm membrane, which has one side in contact with an extract solution of *Vicia angustifolia*. However, an addition of a *V. hirsuta* extract to the medium strongly inhibited the salivary sheath formation. A specific probing deterrent was isolated from a *V. hirsuta* extract by monitoring the inhibitory effect, and identified as (*E*)-2-methyl-2-butene-1,4-diol 4-*O*-β-D-glucopyranoside. A mixture of the glycoside and the stimulatory *V. angustifolia* fraction in the same equivalency found in plants significantly decreased the probing activity in *M. crassicauda*. Since the stylet insertion process is a crucial step for the aphid's settlement on a plant, the glycoside seems to act as an effective chemical barrier for *V. hirsuta*.

Keywords: Aphid; *Megoura crassicauda*; *Vicia hirsuta*; Fabaceae; Probing deterrent; Host selection; Allomone; (*E*)-2-methyl-2-butene-1,4-diol 4-*O*-β-D-glucopyranoside; Hemiterpene glycosides

1. Introduction

The bean aphid, *Megoura crassicauda* Mordvilko, feeds selectively on a variety of fabaceous plants in the genus *Vicia*, such as broad bean (*Vicia faba* L.) and narrow leaf vetch (*Vicia angustifolia* L.). The host selectivity of oligophagous aphids is controlled by specific chemicals in their host plants (Van Emden, 1972; Klingauf, 1987). After landing on a plant, the aphid inserts its stylet into the internal tissues and senses the chemicals contained within (Montllor, 1991 and refs. therein). This information determines whether the aphid leaves the plant or continues to probe further (Van Emden, 1972). During the penetration into the host plant tissue towards the phloem, aphids produce proteinous salivary sheaths (Miles, 1965). The same stylet sheaths can be observed on a stretched Parafilm M membrane, which is in contact with a solution of host plant extract (Mittler and Dadd, 1965). We have recently characterised two acylated flavonol glycosides [quercetin 3-*O*-α-L-arabinopyranosyl-(1 → 6)-(2″-*O*-(*E*)-*p*-coumaroyl)-β-D-galactopyranoside and quercetin 3-*O*-α-L-arabinopyranosyl-(1 → 6)-(2″-*O*-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside] as the specific probing stimulants of *M. crassicauda*, to induce the salivary sheath formation. This suggests that the two compounds are possible host finding cues prior to ingestion of the phloem sap (Takemura et al., 2002).

In the course of this study, we noticed that the tiny vetch, *Vicia hirsuta* (L.) Gray, was never attacked by *M. crassicauda*, while dense colonies of the aphids were commonly observed on a very closely related species, *V. angustifolia*. Both the plant species are common vinous weeds that grow closely together, often tangling with each other during the spring season (April to early June) in Japan. Although the seedpods of *V. hirsuta* are more hairy

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than those of *V. angustifolia*, the surface structure of the stem, on which the aphids would colonise, did not appear different between the two species under a microscope. When the aphids were exposed to a *V. hirsuta* plant in the laboratory, they displayed a short-term probing on the stem, but eventually left the plant. This suggests that the aphids were capable of sensing some odd chemicals in the internal tissues of *V. hirsuta*. An extract of *V. hirsuta* was found to deter the probing behaviour of the aphids when added to the solution containing the positive stimulants in feeding tests using the parafilm membrane. We report the isolation and structural elucidation of a specific probing deterrent in *V. hirsuta* against *M. crassicauda.*

2. Results and discussion

When freshly cut stems from both *V. angustifolia* (host) and *V. hirsuta* (non-host) were placed together in a cage, the introduced adult aphids (apterous or alatae) settled selectively on *V. angustifolia* and started feeding on the stems producing a large quantity of honeydew within few hours. The aphids seemed to conduct some trial probing on *V. hirsuta* as well, but they left the plant within a short period. *M. crassicauda* actively displayed probing behaviour toward the crude aqueous extracts of *V. angustifolia*, depositing a substantial number of thick stylet sheaths (thickness, approx. 10 μm; length > 100 μm) on a parafilm membrane at a concentration of 1.0 g fresh leaf equivalent/ml (gle/ml) (Takemura et al., 2002). However, the aphid deposited much fewer stylet sheaths on the parafilm in contact with a crude aqueous extract of *V. hirsuta* (Table 1). When a mixture of both *V. angustifolia* and *V. hirsuta* extracts was supplied in an equivalency (1.0 gle/ml each), the probing activity decreased significantly (Table 1). This suggests the presence of a deterrent (or masking) substance(s) that interfered with the production of salivary sheaths. The probing deterrent assay was performed by mixing *V. hirsuta* fractions (1.0 gle/ml unless otherwise specified) in a stimulative fraction of *V. angustifolia* (1.0 gle/ml) as shown in Table 1.

An aqueous extract of *V. hirsuta* was applied to an ODS column with increasing concentrations of methanol in water as eluant to give six fractions. These were eluted with water, 10% methanol, 20% methanol, 40% methanol, 60% methanol and 80% methanol in water, respectively. The 10% methanol eluate exhibited a significant probing deterrent activity almost equivalent to that of a crude extract of *V. hirsuta* (no significant difference from a mixture of crude *V. hirsuta* extract + *V. angustifolia* extract, Mann–Whitney *U* test) (Table 1). The active eluate was further separated by a reversed-phase HPLC and a single deterrent compound **1** was isolated as an amorphous solid (yield: 370 μg/g leaf). Compound **1** clearly suppressed the stimulant activity of *V. angustifolia* extract at doses of 1.0 and 2.0 g.l.e, but not at a 0.5 g.l.e. dose (Table 1).

Compound **1** was deduced to possess the molecular formula of $C_{11}H_{20}O_7$ (mw 264) from the electro-spray ionization (ESI) mass spectra (positive *m/z* 287 $[M + Na]^+$; 303 $[M + K]^+$), although $[M + H]^+$ was not observed. An enzymatic hydrolysis of compound **1** with β-glucosidase yielded 2-methyl-2-butene-1,4-diol (**2**) and D-glucose. Compound **2** was determined to possess the (*E*)-configuration by comparing both geometrical isomers derived from mesaconic acid (*trans*) and citraconic acid (*cis*), respectively. The ^{1}H NMR spectrum of **1** (Table 2) exhibited signals arisen from a methyl (δ 1.69), two methylenes (δ 3.96, 2H; δ 4.28 and 4.39, 2H) and an olefinic methine (δ 5.36) of a 2-methyl-2-butene-1,4-diol moiety. A glucopyranosy moiety was found to be attached to one of the primary alcohol to form a β-configuration. The linkage of glucose to the hemiterpene diol

Table 1
Probing responses of *Megoura crassicauda* to solutions of *Vicia* plant extracts and mixtures with various fractions and compound **1**

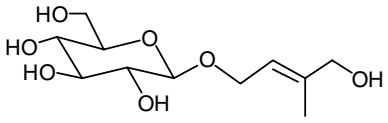
Sample	Probing activity (points) (*N*)
Distilled water	10.5 ± 5.0 (10)[a]
V. angustifolia (*Va*) extract [host, control]	48.2 ± 6.1 (43)
V. hirsuta extract [non-host]	9.1 ± 4.3 (10)[a]
Va ext. + *V. hirsuta* ext.	7.5 ± 3.3 (10)[a]
Va ext. + Fr. 1	45.1 ± 9.0 (9)
Va ext. + Fr. 2	12.0 ± 5.6 (9)[a]
Va ext. + Fr. 3	20.9 ± 5.8 (9)
Va ext. + Fr. 4	41.3± 10.4 (9)
Va ext. + Fr. 5	34.2 ± 6.3 (9)
Va ext. + Fr. 6	35.7 ± 12.3 (9)
Va ext. + Compound **1** (0.5 gle/ml)	22.4 ± 6.9 (10)
Va ext. + Compound **1** (1.0 gle/ml)	17.1 ± 5.0 (10)[a]
Va ext. + Compound **1** (2.0 gle/ml)	13.8 ± 3.4 (10)[a]

Probing activity represents the average frequency of stylet sheath formation.
Dose (gle = gram leaf equivalent): all extracts and fractions were tested at 1.0 gle/ml, except for compound **1** (0.5–2.0 gle/ml).
[a] Significantly different from control *Vicia angustifolia* crude extract (1.0 gle/ml) at $p = 0.05$ using the Mann–Whitney *U* test.

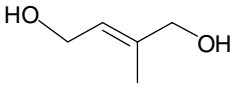
Table 2
^{1}H NMR spectral data for compound **1**, (*E*)- and (*Z*)-2-methyl-2-butene-1,4-diols (CD_3OD, 400 MHz)

Position	Compound **1**	(*E*)-diol (**2**)	(*Z*)-diol
Diol			
1	3.96 *s*	3.93 *s*	4.085 *s*
3	5.36 *br. t* (6.8)	5.59 *br. t* (6.6)	5.47 *t* (6.8)
4	4.28 *m*	4.13 *d* (6.3)	4.11 *d* (8.0)
	4.39 *dd* (12.1, 6.2)		
5	1.69 *s*	1.67 *s*	1.80 *s*
Glucosyl			
1′	4.29 *d* (8.0)		
2′	3.17 *m*		
3′	3.34 *m*		
4′	3.25 *m*		
5′	3.31 *m*		
6′	3.67 *d* (11.7, 4.9)		
	3.87 *br. d* (11.7)		

was determined by the heteronuclear multiple bond correlation (HMBC) spectrum, where correlation from H-1′ (δ 4.29) of glucose to the C-4 (δ 66.2) of diol, and from H-4 (δ 4.39) to the C-1′ (δ 103.0) of glucose were observed. This indicated that the glucose moiety was linked to the C-4 of 2-methyl-2-butene-1,4-diol. Thus, compound **1** was determined as (*E*)-2-methyl-2-butene-1,4-diol 4-*O*-β-D-glucopyranoside. Compound **1** appeared to be a novel compound from plants, although its positional isomer (*E*)-2-methyl-2-butene-1,4-diol 1-*O*-β-D-glucopyranoside (**3**) and (*Z*)-isomer of **3** have been known from other plants (Nicoletti et al., 1992; Kitajima et al., 1998; Zhu et al., 1998). Compounds **2** and **3** were also found in fraction 2 as minor components, but the compounds did not show any significant deterrent effect at the equivalent dosage found in the plant.



Compound **1**



Compound **2**

It is intriguing that a novel hemiterpene glycoside exhibits probing deterrent activity against the aphid. Since the stylet insertion process is a crucial step for the aphid's settlement on a plant (Klingauf, 1987; Montllor, 1991), the glycoside **1** seems to act as an effective chemical barrier for *V. hirsuta*. At the initial step of probing, *M. crassicauda* inserted the stylet 'intercellularly' through the epidermis and mesophyll of host *Vicia* plant. The compound may block the stylet penetration toward the phloem during gustation, if the substance is detected along the intercellular path, although we do not know the distribution of **1** in the plant. Further support for the feeding preference of the aphids between these two closely related *Vicia* species is as follows: firstly, the specific allelochemical is provided in the absence of the probing deterrent **1** of the host *V. angustifolia* and secondly, the lack of the specific probing stimulants (acylated flavonol glycosides previously identified from the host *V. angustifolia*, Takemura et al., 2002) in the non-host plant, *V. hirsuta*.

A series of plant secondary metabolites, including terpenoids (Asakawa et al., 1988), phenolics (Montgomery and Arn, 1974; Dreyer and Jones, 1981; Dreyer et al., 1981; Jones and Klocke, 1987), alkaloids (Corcuera, 1984) and hydroxamic acid derivatives (Argandona et al., 1983; Givovich and Niemeyer, 1995), have been reported as feeding deterrents in several aphid species. However, the actual role of these allelochemicals as probing deterrents, ingestion deterrents or toxicants in many cases remains unclear due to the complexity of host assessment process by aphids plus the lack of knowledge related to the distribution of the chemicals within the plant tissues (Montllor, 1991). A systematic study on the mechanisms of host and non-host recognition of aphids within closely related plant taxa (or cultivars) might provide us a new control technique against pest aphids of economic importance.

3. Experimental

3.1. General

Optical rotation was measured with a JASCO DIP-370 spectropolarimeter. The MS data were recorded with a Shimadzu LCMS-2010A using a reversed phase column (Mightysil RP-18GP, 75 mm × 3 mm i.d.) eluted with 10-100% MeOH in H_2O (0.2 ml/min) with an electro-spray ionization (ESI)-positive mode. ^1H and ^{13}C NMR spectra were measured with a Bruker AC400 FT-NMR spectrometer (400 MHz) with TMS as an internal standard. The letters *s*, *d*, *t* and *m* represent singlet, doublet, triplet, and multiplet, respectively, and coupling constants are given in Hz.

3.2. Plant material

Arial parts of *V. hirsuta* were collected in Sakyo-ku, Kyoto, Japan in April 2003, and identified by Dr. Reiichi Miura of Kyoto University. A voucher specimen is deposited at the Herbarium of Pesticide Research Institute, Kyoto University.

3.3. Extraction and isolation of deterrent

Fresh leaves and stems of *V. hirsuta* (300 g) were extracted with EtOH–H_2O (9:1, v/v) three times. The combined ethanolic solution was evaporated in vacuo, and the residue (30.30 g) was dissolved in H_2O (600 ml) and partitioned four times with hexane (400 ml each). The crude aqueous extract (14.30 g) was applied to a reversed phase column (200 g of Cosmosil 140C18-OPN, Nacalai tesque, 310 × 50 mm i.d.) eluted (2 L each) in sequence (yields of dry materials given in the parentheses) with H_2O (8.69 g), MeOH–H_2O (1:9) (0.40 g), MeOH–H_2O (1:4) (0.17 g), MeOH–H_2O (2:3) (0.30 g), MeOH–H_2O (3:2) (0.41 g) and MeOH–H_2O (4:1) (0.05 g). MeOH–H_2O (1:9) eluate was subjected to preparative HPLC using a reverse phase column (YMC-Pack Pro C18 250 × 10 mm i.d.), eluting with MeOH–H_2O (1:9) at flow rate of 2.0 ml/min. Active fraction was found at a retention time (R_t) range of 18.4–21.4 min, which was further subjected to the same column eluting with MeOH–H_2O (7.5:92.5) at a flow rate of 2.0 ml/min. Compound **1** was isolated at a $R_t = 26.0$ min. The yield of compound **1** from 100 g of

the leaves was 37 mg. Compound **2** was detected at $R_t = 21.5$ min in a small quantity under the latter HPLC condition.

3.4. (*E*)-2-Methyl-2-butene-1,4-diol 4-*O*-β-D-glucopyranoside (*1*)

Colourless solid. $[\alpha]_D^{17}$ −41.8 (10% MeOH in water; *c* 1.00); LCMS (ESI-positive) *m*/*z* (relative intensity): 287 $[M + Na]^+$ (100), 303 $[M + K]^+$ (52). For ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3.

3.5. Enzymatic hydrolysis of compound *1*

Compound **1** (26.4 mg) and β-glucosidase (from almond, Wako Pure Chemical Industries Ltd., 193 units, 5.2 mg) was dissolved in 1 ml of AcOH–NaOAc buffer (1 ml) and incubated for 4 h at 37 °C. The reaction mixture was acidified with 0.5 N HCl, filtered (syringe filter unit, Millex), and passed through a reversed phase column (10 g, Cosmosil 140C18-OPN, Nacalai tesque) eluted with water (100 ml). The water eluate was separated by HPLC (Cosmosil AR-II, 200 × 15 mm i.d.) eluting with MeOH–H_2O (1:4) in H_2O (2.0 ml/min) and D-glucose (15.3 mg $[\alpha]_D^{17} = +43.4$) and (*E*)-diol (**2**) (7.1 mg) were isolated at $R_t = 6.6$ and 16.8 min, respectively.

3.6. Preparation of (*E*)- and (*Z*)-2-methyl-2-butene-1,4-diols

(*E*)-2-methyl-2-butene-1,4-dioic acid dimethyl ester was prepared from mesaconic acid (Tokyo Chemical Industries Co. Ltd.) by refluxing in a mixture with methanol and benzene (3:1) in the presence of a catalytic amount of *p*-toluenesulfonic acid. The ester was reduced to (*E*)-diol (**2**) by treating with $LiAlH_4$ in Et_2O. Likewise, (*Z*)-diol was obtained from citraconic acid (Aldrich Chemical Co. Ltd.) via esterification and $LiAlH_4$ reduction. The ^1H and ^{13}C NMR spectroscopic data are given in Tables 2 and 3.

Table 3
^{13}C NMR spectral data for compound **1**, (*E*)- and (*Z*)-2-methyl-2-butene-l,4-diols (CD_3OD, 100 MHz)

Position	Compound **1**	(*E*)-diol (**2**)	(*Z*)-diol
Diol			
1	68.0	68.2	61.3
2	140.9	138.7	139.1
3	121.7	125.0	127.7
4	66.2	59.2	58.6
5	13.9	13.7	21.5
Glucosyl			
1′	103.0		
2′	75.1		
3′	78.2		
4′	71.7		
5′	78.1		
6′	62.8		

3.7. Bioassay

Adults of *M. crassicauda* (apterous viviparae) were starved for 2 h; and five insects were introduced in a glass tube fitted with a parafilm membrane in contact with either a test solution (0.4 ml) or distilled H_2O (control), and allowed to probe through the membrane for 24 h under laboratory conditions (25 °C, 16L:8D) (Takemura et al., 2002). The number of stylet sheaths deposited on the parafilm membrane was observed under a microscope after being stained with a red fuchsin basic solution. The probing activity was scored by frequency of stylet sheath formation as described previously (Takemura et al., 2002).

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