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Attractant and Oviposition Stimulant of *Crataeva religiosa* Forst. to *Pieris rapae*

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Abstract: We were aimed to confirm the oviposition preference of *P. rapae* against *C. religiosa* (Capparidaceae plant) and cabbage (Brassicaceae plant) and then analyzed the AITC and sinigrin contents and myrosinase activity in these plants to clarify the attractant and oviposition stimulant properties of *C. religiosa* and cabbage to *P. rapae*. We analyzed the attractant and oviposition stimulant of a Brassicaceae plant, *Brassica oleracea* var. *capitata* L. (cabbage) and a Capparidaceae plant, *Crataeva religiosa* to *Pieri srapae* crucivora Boisduval by the oviposition preference test. The oviposition preference of *P. rapae* was the highest in cabbage, followed by *C. religiosa*, but the lettuce (control plant) was rarely selected. Allylisothiocyanate (AITC), which is assumed to be one of the attractant and sinigrin as oviposition stimulants were detected in cabbage using GC-MS. Their compounds were not detected in *C. religiosa*, but the higher content of methylisothiocyanate (MITC) that is analogous to AITC was found. Myrosinase activity was analyzed by HPLC and detected in cabbage and *C. religiosa*, but not in lettuce. Glucosinolate of sinigrin analogous in *C. religiosa* fractionated by HPLC was decreased by myrosinase treatment and then produced MITC. We identified that this compound was glucocapparin as a precursor of MITC by LC-MS. These results revealed that *C. religiosa* have a glucosinolate-myrosinase metabolic system and pathway.

Key words: *Crateva religiosa*, brassicaeae plants, *Pieris rapae*, glucosinolate, isothiocyanate, oviposition

INTRODUCTION

Recently, considering environmental pollution and the safety of food, consumers are strengthening the concern for organic product and chemical-free vegetables and producers are doing more organic farming. Alternative biological pesticides are thus being used to replace agricultural chemicals.

Brassicaceae plants, including many types of important vegetable crops, are extensively damaged by the *P. rapae crucivora* Boisduval. *P. rapae* specifically search for Brassicaceae plants and oviposits on the leaves of these plants (Sato *et al*., 1999). It is known that *P. rapae* utilize sinigrin as an oviposition stimulant in Brassicaceae plants (Huang and Renwick, 1994; Traynier and Truscott, 1991). Most butterflies have an oviposition preference and choose suitable plants upon which to lay eggs; it is thought that they respond to chemical compounds in host plants as a cue to search for a place to lay eggs (Honda, 1995). Glucosinolates (sinigrin, etc.) are plant secondary products as particular components of the family Brassicaceae, which have a glucosinolate-myrosinase system. When tissue disruption occurs due to pest or pathogen, glucosinolates are hydrolyzed with myrosinase and produce a volatile compound, (AITC: allylisothiocyanate), playing a role as defense to other pest (Griffiths *et al*., 2001; Tsao *et al*., 2002; Bridges *et al*., 2002; Tolra *et al*., 2000; Kushad *et al*., 1999). Bridges *et al*. (2002), Finiguerra *et al*. (2001) and Tsao *et al*. (2002) reported that AITC was one of a factor that *P. rapae* search for Brassicaceae plants.

In our previous study (Huang and Renwick, 1993), it was recognized that *P. rapae* was attracted to *C. religiosa* in the Capparidaceae plant, *C. religiosa* is the same order and the different family from Brassicaceae plants. Also, Fahey *et al*. (2001) reported that a sinigrin analogue contained in the subfamily Cleomoideae in the Capparidaceae plant. In this study, we confirmed the oviposition preference of *P. rapae* against *C. religiosa* (Capparidaceae plant) and cabbage (Brassicaceae plant). In addition, we analyzed the AITC and sinigrin contents and myrosinase activity in these plants to clarify the attractant and oviposition stimulant properties of *C. religiosa* and cabbage to *P. rapae*.

MATERIALS AND METHODS

This study was conducted at the Meiji University greenhouse from July 1st to December 25th 2006.

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Plant materials: Seedings of cabbage (*Brassicacea oleracea* var. *capitata* L.) of the Brassicaceae plant and lettuce (*Lactuca sativa* L.) of the Asteraceae plant were grown in the Meiji University greenhouse from July 6th to September 24th 2006 according to cultural guidelines approved by the systematized techniques of agriculture (Iwama, 1974). *Crataeva religiosa* Forst. (Brassicales: Capparidaceae) were obtained from the Museum of Insects in Hiroshima City Forest Park.

Insect: Gravid adult females of Pieris rapae crucivora Boisduval were collected at cabbage patches in Kanagawa prefecture. They were released in a cage (1.6×1.6×1.8 m) covered by a white shading net in the greenhouse and for 24 h oviposited on potted cabbages. An approximately 10% sucrose solution was provided for feeding. The potted cabbages with deposited eggs were placed in natural conditions in the greenhouse until hatching. The hatched larvae were reared on cabbage in a plastic case (10×20×7 cm) at 25°C under a 16L: 8D photoperiod until adult emergence. The adults were kept in a cage in the greenhouse until use in the experiments.

Oviposition preference of *P. rapae*: The first experiment was conducted in the cage of the greenhouse from September 25 to November 25, 2006. Each of three combinations of lettuce (negative control), *C. religiosa* and cabbage of nearly the same size were placed in the cage. Ten butterflies were then released in the cage for 24 h and the number of eggs counted at each plant. Oviposition (%) was calculated using the equation below. The experiment was carried out using three replications.

Quantitative and identification of glucosinolates: The intact glucosinolate was extracted according to Tolra *et al.* (2000). The glucosinolate was extracted from 5 g of *C. religiosa* with 70% aqueous methanol in a boiling water bath for 10 min. After cooling and centrifugation, the proteins of the supernatant were precipitated with a solution containing lead acetate and barium acetate. The tube was stopped and allowed to stand on the water-bath for 10 min. During this time the tube was vigorously shaken twice. The tube was centrifuged and the supernatant transferred to a sample tube. This solution was used for desulfatedglucosinolate extraction and HPLC-MS analysis.

Desulfatedglucosinolates were analyzed according to Kushad *et al.* (1999). Desulfation of the glucosinolate was performed on DEAE anion exchange columns. A 1 mL of the intact glucosinolate solution was layered on a DEAE Sephadex A-25 column. Glucosinolates were desulfated with arylsulfatase while on the column by 10 units of sulfatase suspended in 500 μL of distilled water to the column and then capped for 18 h. The desulfatedglucosinolate were eluted from the column with 2 mL of water. The eluted solution was used on HPLC-MS analysis. The experiment was carried out using three replications.

Liquid chromatograph-mass spectrometry analysis: HPLC-MS was performed with a Shimadzu LC-MS-2010EV/A. The HPLC-UV analysis was carried out on a Shimadzu CBM-20Alite HPLC with a UV detector using a Wako WS5C18 column (4 mm i.d. ×200 mm). The column was maintained at 40°C. The detection wavelength was 228 nm. The flow rate was 1 mL min^{-1}. The HPLC-MS condition for glucosinolate analysis and the gradient condition are shown in Table 1 and 2.

Myrosinase activity: All procedures were undertaken at 4°C. Five grams of *C. religiosa* leaf sample, all of which were stored at -80°C, were homogenized in buffer A (50 mM Mops-NaOH [pH 7.5], 1 mM Na_2EDTA, 2.5 mM DTT and 0.05% (v/v) Triton X-100) and then centrifuged at 12000 rpm for 20 min. The supernatant liquid was passed through a PD-10 column and eluted with buffer B (50 mM Mops-NaOH [pH 7.5], 0.5 mg mL^{-1} BSA, 2.5 mM DTT and 0.05 % (v/v) Triton X-100). The eluate was an enzyme solution. Enzyme activity was determined using sinigrin as the substrate. Fifty microliters of enzyme solution were reacted in a mixture including 0.5 mL of 0.2 M phosphoric acid buffer solution (pH 7.0), 0.1 mL of 10 mM ascorbic acid, 0.15 mL of distilled water and 0.2 mL of 1 mM sinigrin at 37°C for 10 min. The reaction was stopped by heating at 100°C for 10 min. The mixture was passed through a 0.45 μm chromatodisc (Millipore Co., Ltd. Japan). The HPLC analysis was performed under the same conditions as the quantitative analysis of glucosinolate. The experiment was carried out using three replications.

Table 1: LC-MS condition for glucosinolate analysis

Condition	Values	Condition	Values
Column	Wako WS5C18 (4×200 mm)	Ionization	Electrospray, Negative ion
		Fragmentor	155 V
Eluent	Gradient	Nebulizer	N_2 (35 psi)
Flow rate	1 mL min^{-1}	Drying gas	N_2 (1.5 L min^{-1}, 350°C)
Oven temperature	40°C	V-cap	4500 V
Injection volume	100 μL	SIM ion	m/z 254, 163, 145, 41

Table 2: Composition of the mobile phase used in the HPLC for separation of glucosinolates

Time (min)	Water (%)	25% ACN[Z] (%)	Gradient
Initial conditions	100	0	-
5.0	96	4	Linear
30.0	60	40	Linear
35.0	60	40	-
35.1	100	0	Step
45.0			Inject next sample

[Z]Acetonitlile

Quantitative analysis and identification of Isothiocyanate (ITC): Solid-phase micro extraction (SPME) was performed with a 100 μm SPME fiber (Supelco Inc., Bellefonte, PA) coated with poly dimethylsiloxane (DVB/PDMS). For headspace SPME sampling, 1.01 mL of mixture including 0.5 mL of 0.2 M phosphoric acid buffer solution (pH 7.0), 0.1 mL of 10 mM ascorbic acid, 0.15 mL of distilled water, 0.2 mL of glucosinolate fraction solution, 0.01 mL of 1000 ppm cyclohexanol (internal standard), 0.1 mL of myrosinase solution or distilled water (control) at 37°C for 10 min after myrosinase hydrolyze was put into a 4 mL vial and closed with a hole cap faced with PTFE/white silicone; the septum was then equilibrated at 40°C for 10 min. The fiber was exposed to headspace volatiles for 40°C at 10 min. The fiber was then pulled into the sample vial, which was transferred directly to the GC injector. The analyses were desorbed from the SPME coating at 250°C for 3 min in the injection port of the GC operated in the splitless mode.

A Shimadzu GC-MS QP2010 (Shimadzu Co., Ltd., Kyoto, Japan) was used to identify the isothiocyanate in *C. religiosa*. The injector, interface and ion source temperatures were 230°C. The column was a DB-Wax fused silica capillary column (60 m×0.25 mm i.d.; J and W Scientific, Folsom, CA) and the oven temperature was held at 40°C for 10 min and then increased by 3°C min^{-1} to 220°C, at which it was held for 30 min. The resulting GC-MS data for the volatile compounds were identified by comparison of their mass spectra with those of a corresponding reference standard and those listed in databases NIST 27 and 147 (Shimadzu Co., Ltd., Kyoto, Japan). The experiment was carried out using three replications.

Statistical analysis: Analysis of variance was performed on data between and within the groups. The number of eggs laid on each plant was statistically analyzed by a chi-square test under the null hypothesis that the probabilities of *P. rapae* ovipositing on either plant were equal:

$$\text{Oviposition (\%)} = 100\times\frac{\text{The No. of eggs for one plant}}{\text{The total No. of eggs for both plants}}$$

Mean separation of contents of glucosinolate and isothiocyanate and myrosinase activity was determined by Turkey-Kramer test at p = 0.05 and standard division of the mean (SD).

RESULTS AND DISCUSSION

The oviposition preference of *P. rapae* to cabbage, *C. religiosa* and lettuce are shown in Fig. 1. *P. rapae* significantly preferred cabbage (99%, χ^2 test, $\chi^2 = 106.11$, $p<0.001$) and *C. religiosa* (93%, $\chi^2 = 21.56$, $p<0.001$) compared with lettuce. Although *P. rapae* predominantly oviposited on cabbage (80%) rather than on *C. religiosa* (20%), it was clearly indicated that *P. rapae* oviposited on *C. religiosa*. These results supported our previous result. (Ikeura *et al*., 2008)

Next, we measured AITC, sinigrin and myrosinase activity in used plants (Fig. 2a-c). The AITC and sinigrin contents and myrosinase activity in cabbage highly selected by *P. rapae* were 2.61 μmol g^{-1}, 15.51 μmol g^{-1} and 2.51 μmol/m/gFW, respectively. The myrosinase activity of *C. religiosa* selected by *P. rapae* was 3.41 μmol/m/gFW, but AITC and sinigrin were not detected at all. However, we found that MITC, an analogue of AITC, was abundantly contained at 12.61 μmol g^{-1} in *C. religiosa*. In lettuce, these compounds and myrosinase activity were not detected at all. These results demonstrated that the glucosinolate-myrosinase system exists in *C. religiosa*, therefore we presumed its glucosinolate-myrosinase pathway (Fig. 3). The pathway is initiated by the conversion of alanine to aldoxime. Biosynthetic steps after aldoxime formation are believed to involve conversion to a thiohydroximate, introduction of thioglucoside sulfur from cysteine and S-glycosyl transfer from UDP-glucose. S-glycosylation of thiohydroximate is catalyzed by thiohydroximateglucosyltransferase and results in a

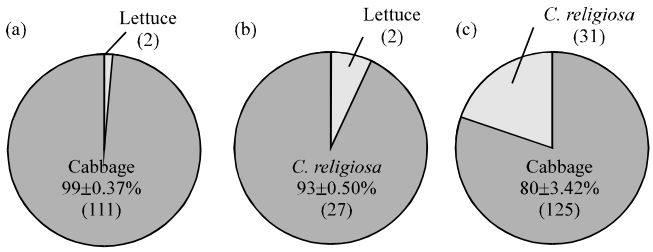

Fig. 1: Oviposition preference of *P. rapae* to Cabbage, *C. religiosa* and lettuce. (a) cabbage vs. lettuce, (b) *C. religiosa* vs. lettuce and (c) cabbage vs. *C. religiosa*. Numbers in parentheses indicate the average number of oviposited eggs

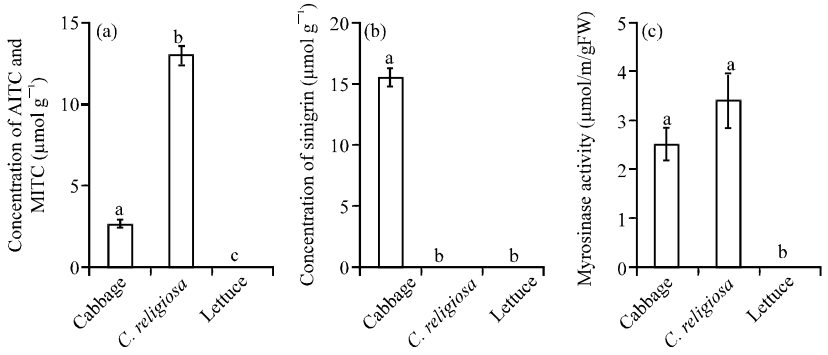

Fig. 2: Contents of both of isothiocyanate and sinigrin and myrosinase activity in cabbage, *C. religiosa* and lettuce. (a) AITC and MITC, (b) sinigrin, (c) myrosinase activity. Parallel bars represent the standard deviation of the mean. The same letters are not significantly different at $p<0.001$ by Turkey-Kramer test

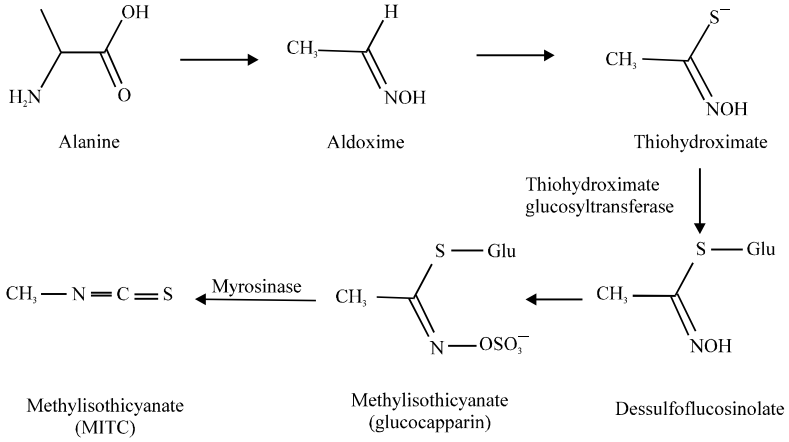

Fig. 3: Pathway for biosynthesis of glucocapparin and MITC

desulfoglucosinolate. The final step in glucosinolates formation is the sulfation of desulfoglucosinolates catalyzed by desulfoglucosinolatesulfotransferase. Then, the glucosinolate was hydrolyzed by myrosinase and methylisothiocyanate (MITC) was produced (Fahey *et al*., 2001). To confirm the glucosinolate-myrosinase system in *C. religiosa*, the glucosinolate fraction extracted from *C. religiosa* using HPLC was treated with myrosinase enzyme and then the glucosinolate and MITC contents were measured (Table 3). The content of glucosinolate decreased rapidly from 37-13 μmol g^{-1} with myrosinase treatment and we found MITC in this fraction, these data indicated to be the glucosinolate-myrosinase system in *C. religiosa*, which the glucosinolate was hydrolyzed by myrosinase and MITC was produced. These results were supported by the report that MITC was contained in *Boscia senegalensis* Pers. of the Capparideae plants (Seck *et al*., 1993).

Next, the mass spectrum of glucosinolate which was analogous to sinigrin in *C. religiosa* and obtained by

Table 3: The contents of glucocapparin and mitc after myrosinase treatment

Treatment	Glucocapparin (μmol g^{-1}±SD)	MITC (ppm±SD)
Non-treatment	37.32±4.91a[z]	0.00b
Myrosinase	13.60±4.29b	7.60±1.89a

[z]Values within a row with different letters are significantly different (Turkey-Kramer test, $p<0.05$)

HPLC/ESI-MS is shown in Fig. 4. Negative ion ESI in the full scan mode indicated that the glucosinolate had mass spectrum of 333 (ions at m/z 332, [M-H]-) (Fig. 4a). Supporting these data, desulfatedglucosinolate was obtained by mass spectrum of [C6H11O5-] (m/z 163), [C6H9O4-] (m/z 145) and [CH3CN-] (m/z 41) ion in the selected ion mode (Fig. 4b). These mass spectrum corresponded to the mass spectrum of fragment ion (m/z 254, 163 and 145) reported by Kiddle *et al*. (2001). From these results, we identified the glucosinolate as glucocapparin (methylglucosinolate). There has been no report of the oviposition preference of *P. rapae* and glucocapparin and MITC in *C. religiosa*, however Fahey *et al*. (2001) reported that glucocapparin contained

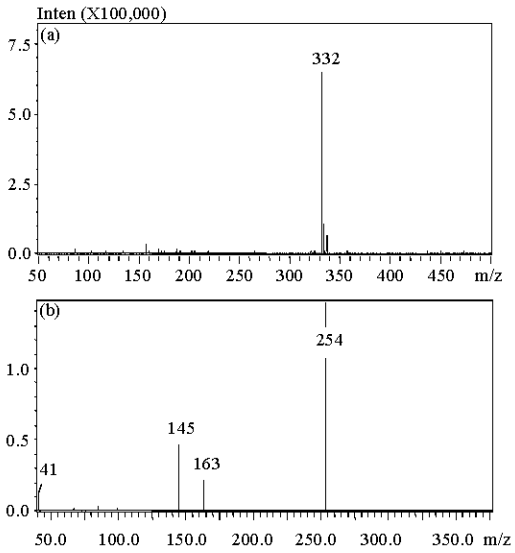

Fig. 4: Negative ion electrospray mass spectra of intact (a) glucocapparin and (b) desulfatedglucocapparin

in the subfamily Cleomoideae in the Capparidaceae plant. Hovanitz and Chang, (1963) observed that *P. rapae*oviposited on Cleome. These reports supported our results that glucocapparin presented in *C. religiosa*, in the Capparidaceae plant. Traynier and Truscott (1991) indicated that oviposition stimulants of *P. rapae* were sinigrin, gluconasturtiin and glucobrassicin. Additionally, our results demonstrated that glucocapparin may be a new oviposition stimulant.

Present results elucidated that *P. rapae*oviposited on *C. religiosa* of the Capparideae family and that the glucosinolate-myrosinase system (glucocapparin was hydrolyzed by myrosinase and produced MITC) is presented in *C. religiosa*. It is a strong possibility that MITC is an attractant and glucocapparin is an oviposition stimulant to *P. rapae*. In the future, we will attempt to prove these hypotheses.

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REFERENCES

Bridges, M., A.M.E. Jones, A.M. Bones, C. Hodgson and R. Cole *et al*., 2002. Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. Proc. Biol. Sci., 269: 187-191.

Fahey, J.W., A.T. Zalcmann and P. Talalay, 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry, 56: 5-51.

Finiguerra, M.G., R. Iori and S. Palmieri, 2001. Soluble and total myrosinase activity in defatted *Crambe abyssinica* meal. J. Agric. Food Chem., 49: 840-845.

Griffiths, D.W., N. Deighton, A.N. Birch, B. Patrian, R. Baur and E. Stadler, 2001. Identification of glucosinolates on the leaf surface of plants from the Cruciferae and other closely related species. Phytochemistry, 57: 693-700.

Honda, K., 1995. Chemical basis of differential oviposition by Lepidopterous insects. Arch. Insect Biochem. Physiol., 30: 1-23.

Hovanitz, W. and V.C.S. Chang, 1963. Ovipositional preference tests with pieris. J. Res. Lepidoptera, 2: 185-200.

Huang, X. and J.A.A. Renwick, 1993. Differential selection of host plants by two *Pieris* species: The role of oviposition stimulants and deterrents. Entomol. Exp. Appl., 68: 59-69.

Huang, X. and J.A.A. Renwick, 1994. Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracea*. J. Chem. Ecol., 20: 1025-1037.

Ikeura, H., T. Gomi, N. Momono and Y. Hayata, 2008. Oviposition preference for adult female of the cabbage butterfly in brassicaceae and capparidaceae plants. Japan Soc. Agric. Technol. Manage., 15: 117-122.

Iwama, S., 1974. Systematized Techniques of Agriculture. Rural Culture Association, Tokyo.

Kiddle, G., R.N. Bennett, N.P. Boting, N.E. Davidson, A.A. Robertson and R.M. Wallsgrove, 2001. High-performance liquid chromatographic separation of natural and synthetic desulphoglucosinolates and their chemical validation by UV, NMR and chemical ionisation-MS methods. Phytochem. Anal., 12: 226-242.

Kushad, M.M., A.F. Brown, A.C. Kurilich, J.A. Juvik, B.P. Klein, M.A. Wallig and E.H. Jeffery, 1999. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. Agric. Food Chem., 47: 1541-1548.

Sato, Y., S. Yano, J. Takabayashi and N. Ohsaki, 1999. Pieris rapae (Lepidoptera: Pieridae) females avoid oviposition on *Rorippaindica* plants infested by conspecific larvae. Applied Entomol. Zool., 34: 333-337.

Seck, D., G. Longnay, E. Haubruge, J.P. Wathelet, M. Marlier, C. Gaspar and M. Severin, 1993. Biological activity of the shrub *Boscia senegalensis* (Pers.) Lam. ex Poir. (Capparaceae) on stored grain insects. J. Chem. Ecol., 19: 377-389.

Tolra, R.P., R. Alonso, C. Poschenrieder, D. Barcelo and J. Barcelo, 2000. Determination of glucosinolates in rapeseed and *Thlaspicaerulescens* plants by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 889: 75-81.

Traynier, R.M.M. and R.J.W. Truscott, 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae*. J. Chem. Ecol., 17: 1371-1380.

Tsao, R., C.J. Peterson and J.R. Coats, 2002. Glucosinolate breakdown products as insect fumigants and their effect on carbon dioxide emission of insects. BMC Ecol., 2: 5-5.