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Flavonoids as Feeding Stimulants of the Beetles Attacking the Polygonaceous Plants

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Summary

For understanding of the mechanism of the host selection by 4 species of beetles attacking the Polygonaceous plants, the effects of quercitrin which was isolated and identified in the *Rumex obtusifolius* leaves, the common host of these insects and 5 other related flavonoids on the feeding of these insects were investigated. For the feeding of the strawberry leaf beetle, *Galerucella vittaticollis* BALY which feeds on strawberry leaves besides Polygonaceous plants, quercitrin and rutin were stimulative at a 0.01 M concentration both in combination with and without sucrose. For the feeding of *Gastrophysa atrocyanea* MOTSCHULSKY, *Gallerucida bifasciata* MOTSCHULSKY and *G. nigromaculata* BALY were also stimulated by quercitrin and rutin at the same concentration in the presence of sucrose, but were not effected by these flavonoids alone. These results indicate that these 4 species of beetles were likely to require quercetin glucosides such as quercitrin and rutin which are widely distributed in the Polygonaceous plants, as feeding stimulants in their host selection.

Matsuda and Matsumoto (1975) showed that the strawberry leaf beetle, *Galerucella vittaticollis* BALY, which feeds on plants of the family Polygonaceae and the strawberry, *Fragarria chiloensis*, do not require organic acid components, such as oxalic, malic, tartaric and citric acids, i.e., which are the characteristic taste substances of the Polygonaceous plants, as feeding stimulants in the host selection, whereas *Gastrophysa atrocyanea* MOTSCHULSKY, *Gallerucida bifasciata* MOTSHULSKY and *G. nigromaculata* BALY whose host ranges are restricted to the Polygonaceae require these acids.

In this paper, as the second step towards the understanding of the host plant selection by the strawberry leaf beetle, investigation was initiated to isolate the feeding stimulants present in *Rumex obtusifolius*, one of the host plant of this insect. During the investigation it emerged that *Rumex obtusifolius* leaves contained flavonoid quercitrin. Flavonoids have been known to serve as feeding stimulants for several phytophagous insects (Hamamura et al. 1962; Hedin et al, 1968; Guerra and Shaver, 1969; Zielske et al, 1972; Levy et al, 1974). Therefore,

the effects of quercitrin and 5 related flavonoids as feeding stimulants of the strawberry leaf beetle were further investigated. In addition, the effects of these flavonoids on the feeding of *G. atrocyanea*, *G. bifasciata* and *G. nigromaculata* were subsequently tested.

Materials and Methods

Insect: The strawberry leaf beetles, *G. vittaticollis* used in the experiments were selected from a laboratory culture maintained exclusively on *R. obtusifolius* leaves. *G. atrocyanea* were collected at the larval stage from *R. obtusifolius* and reared on leaves of the same plant until adult. *G. bifasciata* were collected at the adult stage from *R. obtusifolius* and *Polygonum thunbergii*, and reared on *R. obtusifolius* leaves for at least 2–3 days before use for experiments. *G. nigromaculata* were collected at the adult stage from *Polygonum sachalinense* and then reared on the leaves of *P. cuspidatum*.

Bioassy: The effects of respective flavonoids as feeding stimulants were examined in the choice trial test in which nibbling by adult insects on filter papers moistend with test substances was compared with that on papers treated with control substances. The testing chamber was composed of plastic petri dish 9 cm in dia. and 1.5 cm in height. Three 7 cm in dia. filter papers immersed with 2 ml water were placed on the bottom of the petri dish, and the doughnut-like plastic disc (3 cm I.D and 7 cm O.D) was put on the papers. The two pieces of 2 cm square filter paper moistend with the test substance were placed oppositely on the doughnut-like disc, and those immersed in solvent only (control) were there placed oppositely and equidistantly between these.

Filter papers for the assay were prepared as follows: The papers were first adsorbed each with 0.075 ml of methyl alcohol dissolving test substance. Control papers were treated similarly with the same amount of methyl alcohol only. After evaporating off the solvent, each filter papers was moistend with 0.075 ml of water. For the evaluation of possible synergistic effect of sucrose on flavonoid, filter papers treated with methyl alcohol, or methyl alcohol solution containing test substance as mentioned above were further moistend each with 0.075 ml of sucrose solution instead of water. The choice of the 0.5 M sucrose solution as synergist depended on the results in preliminary tests that sucrose had more stimulative effect on the feeding of *G. vittaticollis*, *G. atrocyanea*, *G. bifasciata* and *G. nigromaculata* than fructose and glucose do, and that the 0.5 M concentration of sucrose was more effective than 0.1, 0.2, 0.3 and 0.4 M for these four species.

The insects for assay previously starved in a moist dish were introduced into the test chamber and allowed to feed on the filter papers. Because insects used in these assays had differences in physique and food consumption, test conditions were differed with species as follows: *G. vittaticollis* and *G. nigromaculata*: starvation period, 24 hrs; test duration, 24 hrs; number of released insect, 20. *G. atrocyanea*

and *G. bifascyata*: starvation period, 8 hrs; test duration, 24 hrs; number of released insects, 15. Comparisons were made at three concentration levels of 0.1, 0.01 and 0.001 M, being replicated five times for *G. vittaticollis*. But for the other three species, three replicated tests were run at 0.01 M concentration.

The degrees of feeding response were judged depending on the differences of the condition of the test filter papers nibbled by insects from that of control papers and expressed by a graded number of "S" signs. "N" sign indicated no stimulative effect, and "In" sign demonstrated feeding inhibition of the insects by the test substance. Maximum feeding response was observed and rated as "SSSS" when test insects were allowed to nibble the filter papers each treated with 0.075 ml of 25 % (W/V) extract by methyl alcohol of leaves from *R. obtusifolius* and *P. cuspidatum*, both of which were used for the rearing of the insects. Less stimulating effects were graded proportionately by smaller numbers of "S" signs.

Extraction and Isolation of Feeding Stimulants: The air dried, ground leaves of *R. obtusifolius* (200 g) were extracted with 70% aqueous methyl alcohol (200 ml) on a water bath at 60°C for 3 hrs, and insoluble materials were removed by filtration. The extraction procedures were repeated two more times. After evaporating off methyl alcohol under vacuum from the extract, the obtained aqueous solution was allowed to stand overnight, when the occurred resinous precipitates were removed by filtration. Then the aqueous filtrate was shaken with chloroform to remove any chloroform soluble compounds, and further extracted four times with 500 ml portion of ethyl acetate. The combined ethyl acetate extracts were concentrated to 100 ml under vacuum, and the deposited brown solids were filtered off after cooling. The filtrate was mixed with equal volume of ether, and resulting amorphous yellow precipitates were collected by filtration. The precipitates were dissolved in hot water. After beeing cooled, the aqueous solution was shaked with chloroform once more to remove chloroform soluble compounds. The residue obtained by expelling the solvent was dissolved in a minimum amount of ethyl acetate saturated with water and applied to the top of cellulose powder (200–300 mesh) column (4× 40 cm), which had previously been packed using the same solvent. The chromatography was carried out with ethyl acetate saturated with water and each 10 ml of eluate was collected. Each eluate was subsequently analyzed by paper chromatography with the following solvent: n-butanol-acetic acid-water (4:1:5). The fractions of the eluates giving single spot of a same Rf value on the paper chromatogram were combined, evaporated to dryness and shown to give a yellow powder. The yellow powder was recrystallized from a hot water. The yield was 68 mg. The isolated flavonoid gave yellow shining needles, m.p. 166–167°C. The needles were colored yellow by NaOH, green by $FeCl_3$ and redish violet by HCl-Mg.

Fifteen mg of the flavonoid was hydrolyzed by refluxing for 2 hrs at 100°C with 5% HCl. A yellow precipitate was collected by filtration, washed with water, dried and weighed (9.3 mg). The identification of the isolated aglycone was

confirmed by paper chromatography with three different solvent systems: n-butanol-acetic acid-water (4:1:5); acetic acid-water (15:85); and 100% water. Rf values agreed with those of authentic quercetin in any of the solvent systems tried.

The filtrate obtained after filtration of the hydrolysate of flavonoid in the above experiment was concentrated under vacuum, and dried over NaOH. The sugar was identified by paper chromatography with two solvent systems: n-butanol-acetic acid-water (4:1:2) and n-butanol-pyridine-water (3:2:1.5), as rhamnose based on the comparison of Rf values with those of authentic chemicals. The ratio of aglycone to the glucoside in flavonoid was estimated to be 62 %, which indicates the presence of 1 mole of sugar per mole of aglycone.

The position of the sugar residue in the glucoside was determined as follows: Flavonoid dissolved in methyl alcohol was treated with an excess amount of diazomethane solution in diethyl ether. The mixture was stored several days at room temperature. After vacuum evaporation, the resulting product was hydrolyzed with 5 % HCl. The product was shown to be identical with authentic quercetin-3′,4′,5,7-tetramethyl ether by paper chromatography. This proves the attachment of the sugar at the C-3 position of the aglycone.

Flavnoid was therefore concluded to be quercetin-3-rhamnoside (quercitrin).

Results and Discussion

Feeding Response of G. vittaticollis to Flavonoids: Effects of various flavonoids on the feeding of *G. vittaticollis* are summerized in Table 1. Quercitrin and rutin were slightly active at the concentration of 0.01 M. It is noticeable that more stimulative effects were induced by these chemicals when tested in the presence of 0.5 M sucrose. On the other hand, the aglycone of these flavonoids, quercetin showed an inhibitory effect at 0.01 M concentration both with the presence and absence of sucrose. Myricitrin, myricetin and morin did not show any stimulative effect on the feeding of this insect in the choice tests combined with water, and even with 0.5 M sucrose at the levels of 0.001 M and 0.01 M concentration, but rather acted as deterrents of feeding at the concentration of 0.1 M. It is interesting to notice that also in the latter groups, myricetin, the aglycone of myricitrin showed a somewhat different effect from that of the glucosides.

Comparison of Feeding Response of Four Species of Beetles to Flavonoids: Effects of flavonoids as feeding stimulants of *G. atrocyanea*, *G. bifasciata* and *G. nigromaculata* besides with that of *G. vittaticollis* are summerized in Table 2. Comparisons were made at 0.01 M concentration in this case, depending on the results of Table 1 showed that active flavonoids as feeding stimulants brought the most intensive feeding response to *G. vittaticollis* at this concentration. Quercitrin and rutin were also effective to stimulate the feeding of these three species as in the case of *G. vittaticollis* in combination with sucrose, but these flavonoids were inactive in the absence of sucrose for them, different from the result with *G.*

TABLE 1. *Effects of Various Flavonoids on the Feeding of Galerucella vittaticollis*

Flavonoid	Concentration	Feeding response to flavonoid	
		Flavonoid: H_2O	Flavonoid in 0.5 M sucrose: 0.5 M sucrose
Quercitrin	0.1	In	N
	0.01	S	SS
	0.001	N	S
Quercetin	0.1	In	In
	0.01	In	In
	0.001	N	N
Rutin	0.1	N	N
	0.01	S	SS
	0.001	N	S
Myricitrin	0.1	In	N
	0.01	N	N
	0.001	N	N
Myricetin	0.1	In	In
	0.01	N	N
	0.001	N	N
Morin	0.1	In	In
	0.01	N	N
	0.001	N	N

vittaticollis. Myricitrin was inactive, and quercetin, myricetin and morin acted rather as deterrents to the former three species of beetles, while they did not cause an inhibitory effect to *G. vittaticollis*. It can be pointed out that the flavonoids acting as feeding stimulants of *G. vittaticollis* also stimulate, but less effectively the feeding of *G. atrocyanea*, *G. bifasciata* and *G. nigromaculata*, and that the flavonoids acting as feeding detrrents of the former insect exhibit inhibitory effect at much lower concentration to the latter three species.

Various flavonoids known in the leaves of Polygonaceous plants are listed in Table 3. All of Polygonaceous plant except for three species, i.e., *P. orientale* and *R.*

TABLE 2. *Effects of Various Flavonoids on the Feeding of Gastrophysa*

Flavonoid	Feeding response			
	G. atrocyanea		*G. nigromaculata*	
	0.01 M: H_2O	0.01 M in 0.5 M sucrose: 0.5 M sucrose	0.01 M: H_2O	0.01 M in 0.5 M sucorse: 0.5 M sucrose
Quercitrin	N	S	N	S
Quercetin	In	In	In	In
Rutin	N	S	N	S
Myricitrin	N	N	N	N
Myricetin	In	In	In	In
Morin	In	In	In	In

TABLE 3. *List of Flavonoids Identified in the Leaves of Polygonaceous Plants*

Scientific Name	Flavonoids Detected	References
Polygonum hydropiper	Qu 3-rutinoside (Rutin)	Valentin and Wagner, 1953.
	Qu 3-rhamnoside (Quercitrin)	Hörhammer and Rao, 1954.
	Isorhamnetin-KSO_3 (Persicarin)	Harborne, 1967.
P. aviculare	Qu 3-arabinoside (Avicularin)	Ohata, 1940.
P. polystachum	Qu 3-arabinoside (Avicularin)	Hörhammer et al, 1958.
P. cuspidatum	Qu 3-xyloside (Reynoutrin)	Nakaoki and Morita, 1956.
P. sachalinense	Qu 3-rhamnoside (Quercitrin)	Nakaoki and Morita, 1956.
P. orientale	Ap 8-glucoside (Vitexin)	Hörhammer et al, 1958.
	Lu 6-glucoside (Iso-orientin)	Hörhammer et al, 1958.
P. convolvulus	Qu 3-rutinoside (Rutin)	Hirao, 1954.
Fagopyrum esculentum	Qu 3-rutinoside (Rutin)	Harborne, 1967.
F. cymosum	Qu 3-rutinoside (Rutin)	Kimura and Kijima, 1969.
F. tatarium	Qu 3-rutinoside (Rutin)	Hirao, 1954.
Rheum emodi	Qu 3-rutinoside (Rutin)	Hörhammer and Müller, 1954.
R. pruinosum	Qu 3-rutinoside (Rutin)	〃
R. officinale	Qu 3-rutinoside (Rutin)	〃
R. rhaponticum	Qu 3-rutinoside (Rutin)	〃
R. undulatum	Qu 3-rutinoside (Rutin)	〃
Rumex acetosa	Qu 3-galactoside (Hyperin)	Hörhammer and Votz. 1955.
R. crispus	Cy 3-glucoside	Koukol and Dugger, 1967.
R. eckloniamus	Kaemferol	Tutin and Clever, 1910.

Qu=Quercetin, Ap=Apigenin, Lu=Luteolin, Cy=Cyanidin

crispus and *R. eckloniamus* contain quercetin glucosides in which rutin is most broadly detected. Quercitrin, identified from *R. obtusifolius* in this article, has been known also in *P. hydropiper* and *P. sachalinense* leaves.

The differences between *G. vittaticollis* and the three other species of bettles have also been pointed out in a previous paper (Matsuda and Matsumoto, 1975), in the feeding response to organic acids such as oxalic, malic, tartaric and citric acid which are characteristic components of their host plants. The organic acids do not stimulate the feeding of *G. vittaticollis*, but act as feeding stimulants for the latter species. These results indicate that flavonoids and organic acids, which are major characteristic secondary products of Polygonaceous plants, fill an important role

atrocyanea, Gallerucida nigromaculata and Gallerucida bifasciata

to flavonoid

G. bifasciata		*G. vittaticollis*	
0.01 M: H_2O	0.01 M in 0.5 M sucrose: 0.5 M sucrose	0.01 1M: H_2O	0.01 M in 0.5 M sucrose: 0.5 M sucrose
N	S	S	SS
In	In	In	In
N	S	S	SS
N	N	N	N
In	In	N	N
In	In	N	N

in combination or by themselves in the host selection by the insect attacking these plants. The wider host ranges of *G. vittaticollis* including *F. chiloensis* besides Polygonaceous plant than that of the three other species of beetles may be partly attributable to its higher sensivity of flavnoids and non-sensivity to organic acids, but details still remain to be further investigated.

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