Probing Stimulants from the Rice Plant towards the Smaller Brown Planthopper, *Laodelphax striatellus* (FALLÉN) (Homoptera: Delphacidae)

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Z. Naturforsch. **55c,** 1038–1043 (2000); received August 4/August 29, 2000

Laodelphax striatellus, Probing Stimulant, Rice Plant

When adult males of the smaller brown planthopper, *Laodelphax striatellus* were fed on 2% crude rice (leaf and stem) extract containing 15% sucrose there were characteristic stylet sheaths deposited on parafilm membrane. Further bioassays with the butanol-soluble fr. of the extract revealed that it is highly effective for the insects. When the butanol fr. was charged on an ODS open column and eluted in sequence with 20, 40 and 100% methanol in water, the ODS-40% methanol fr. was shown as the most effective one. Further separation of the ODS-40% methanol fr. resulted in six effective components. These components acted to stimulate very high probing response on *L. striatellus* only when they are combined. This activity was found to be similar to those of the ODS-40% methanol fr. and the original crude rice plant extract. Two of the active components were identified as tricin 5-*O*-glucoside and tricin 7-*O*-glucoside, respectively, through spectroscopic analyses.

Introduction

The smaller brown planthopper, *Laodelphax striatellus*, the white-back planthopper, *Sogatella furcifera* as well as the brown planthopper, *Nilaparvata lugens* are the most notorious rice pests in the East Asian countries including Japan and South Korea. They do not only cause direct damage, by removing plant sap, but are also vectors of the serious rice pathogens. In particular, *L. striatellus* is important as the vector for the rice black-streak dwarf and rice stripe (Michael and Michael, 1991).

The insecticidal control of these planthoppers is becoming extremely difficult due to the rapid development of resistant breaking populations even after the introduction of the high yielding resistant rice varieties to control them (Maxwell, 1977; Grayer *et al.*, 1994). For this reason, the development of a new pest control method based on the chemistry of the characteristic behaviors or habits of these planthoppers is desired. The probing activity which is one of such behaviors is well known to be performed prior to the sucking behavior of the planthoppers during their feeding. Therefore, manipulating the probing behavior, it may have the possibility of the development of a new pest control method. Meanwhile, the probing behavior of the planthoppers is known to be stimulated by certain specific secondary plant chemicals in the host plant (Sogawa, 1982).

In this connection, already the probing stimulants in the rice plant for *N. lugens* have been isolated and identified as eight flavonoid *C*-glycosides (Kim *et al.*, 1985; Besson *et al.*, 1985) and those for *S. furcifera* were reported as two compounds including a flavonoid *O*-glucoside (Adjei-Afriyie *et al.*, 2000). However, the probing stimulants for *L. striatellus* are not isolated and identified yet. This paper, therefore, reports on the isolation and identification of six components from rice plant acting together as probing stimulants towards *L. striatellus*. Two of the six components have been identified through spectroscopic analyses as tricin 5-*O*-glucoside and tricin 7-*O*-glucoside.

Materials and Methods

The LC-MS data were recorded with a VG Quattro, flow injection method with solvent system: acetonitrile : water = 50 : 50 v/v; flow rate 10 µl/min with ESI-negative and ESI-positive modes. The NMR data were obtained through a JEOL JNM-LA400 spectrometer, PMR (400 MHz) and ^{13}C NMR (100 MHz). TMS and TMSP (3-(trimethylsilyl)propionic-2, 2, 3, 3-d_4 acid sodium salt) were used as the internal standards. The letters s, d and m represent singlet, doublet and

Abbreviations: ODS, octa-decanoyl-silicon; HPLC, high performance liquid chromatography; ANOVA, analysis of variance; DMRT, Duncann's multiple range Test; LC-MS, liquid chromatographic mass spectrometry; UV, ultra violet radiation; *Rt.*, retention time.

0939–5075/2000/1100–1038 $ 06.00

multiplet, respectively, and coupling constants (J) are given in Hz.

Insect

Stock colonies of *L. striatellus* were reared successively on rice seedlings at 25–28 °C, relative humidity of 60–70% with a 16 : 8 (L : D) illumination.

Extraction procedure for the rice plant

A quantity of 2.5 kg of fresh stems and leaves obtained from Toyonishiki rice variety which had been cultivated for 14 weeks without the application of pesticides were cut into pieces (10 cm long) and extracted three times with 90% MeOH in water for about 3 days under darkness. The extract was concentrated under vacuo and the residue obtained was further defatted three times with hexane to obtain an aqueous layer (89.8 g) which was topped up to 4490 ml to obtain a 2% equivalent concentration of the "crude rice plant extract".

Bioassay for probing responses

Since it is difficult to distinguish oviposition marks from probing sheaths, adult males instead of females of *L. striatellus* (Mitsuhashi and Koyama, 1975) were starved for 2 hr and used in bioassays. The insects which were introduced into the assay apparatus were given various test solutions with 15% sucrose or only 15% sucrose solution as a control through a parafilm membrane. In this way, the concentration of each test solution was kept at the same concentration as original 2% crude rice plant extract. The pH value of each test solution was adjusted to neutral by adding either an HCl or KOH solution (Auclair, 1965; Sakai and Sogawa, 1976). All treatments were replicated ten times. After 24 hr, probing sheaths deposited on the parafilm were observed under a microscope after being stained with a red fuchsin basic solution. The probing sheaths were classified according to their branching as non-branched, two-branched, three-branched, and more than four-branched, respectively, and assigned coefficients of 1, 2, 3 and 4. The intensity of probing activity was obtained as the total number of points (Kim *et al.*, 1985). Frequency data were subjected to square root transformation before analysis (Sogawa, 1974). All data were analysed by a one-way ANOVA and means were compared by using DMRT.

Isolation of the two tricin glycosides and the other unidentified active components

The "crude rice plant extract" (89.8 g) was dissolved in distilled water (2 *l*) and then extracted with a water-saturated butanol (1.5 l × 3). The bu-

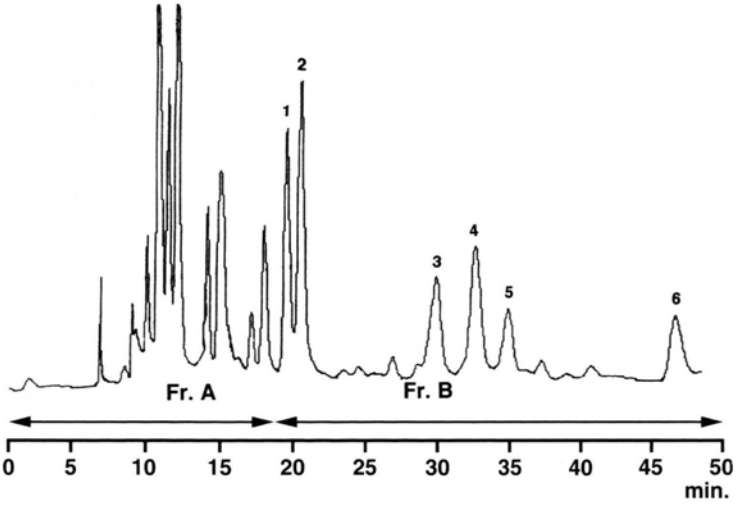


Fig. 1. HPLC profile of the ODS-40% methanol extract showing the main fractions A, B and all the six components **1–6** in fraction B. Obtained by a Cosmosil 5 Ph column (120 mm × 10 mm i. d.) eluted with 20% acetonitrile in water and 1% acetic acid at a flow rate of 2 ml/min and monitored at UV 254 nm.

tanol extract (7.6 g) was chromatographed on an ODS open column (205 mm × 25 mm i.d., 50 g of Chromatorex DM1020T, 100–200 mesh; Fuji Silysia Chemical) with water and 20%, 40% and 100% MeOH to obtain 3.35 g, 2.95 g, and 1.31 g, respectively. The ODS-40% MeOH eluate was separated into two fractions, Fraction A (*Rt* = 0–18.0 min) and Fraction B (*Rt* = 18.0–50.0 min) by reversed phase HPLC (Cosmosil 5 Ph column, 250 mm × 10 mm i.d.), eluting with 20% acetonitrile in water and 1% acetic acid at a flow rate of 2 ml/min and detecting at UV 254 nm. Fraction B was then further separated into components **1** - **6** {**1** (*Rt* = 19.8 min), **2** (*Rt* = 20.8 min), **3** (*Rt* = 30.1 min), **4** (*Rt* = 32.1 min), **5** (*Rt* = 34.9 min) and **6** (*Rt* = 46.5 min)}, respectively, as shown in Fig. 1.

Of these six components, compounds **3** (73.3 mg) and **6** (83.7 mg) were obtained through preparative HPLC from the peaks **3** and **6**, respectively. Through their respective standard curves the actual amounts of compounds **3** and **6** in the 2% equivalent concentration were obtained as 84 and 64 ppm, respectively.

Compound **3**: LC-MS *m/z* (rel. intens.): 493 (9) M^+, 491(31) M^-; PMR 400MHz DMSO-d_6: δ 7.30 (C-2′, 6′, 2H, s), δ 6.85 (C-3, 1H, s), δ 6.83 (C-6, 2H, s), δ 4.75 (C-1″, 1H, d, *J* =7.08Hz), δ 3.90 (C-3′, 5′-*O*-Me, 6H, s), δ 3.79 (C-6″a, 1H, d, *J* =11.5Hz), δ 3.59 (C-6″b, 1H, dd, *J* =11.2Hz, 5.1Hz), δ 3.31 (C-2″, 3″, 4″, 5″, 4H, m).

^{13}C NMR 100MHz DMSO-d_6: δ177.0 (C-4), δ162.8 (C-2), δ161.0 (C-7), δ158.3 (C-5), δ158.5 (C-9), δ148.1 (C-3′, 5′), δ139.4 (C-4′), δ120.4(C-1′), δ108.1 (C-10), δ104.4 (C-2′, 6′), δ106.3 (C-3), δ104.3 (C-6), δ98.5 (C-8), δ104.0 (C-1″), δ77.5 (C-5″), δ75.6 (C-3″), δ73.6 (C-2″), δ69.6 (C-4″), δ60.8 (C-6″), δ56.3 (C-3′, 5′- *O*-Me).

Compound **6**: LC-MS *m/z* (rel. intens.): 493 (17) M^+, 491 (51) M^-; PMR 400MHz DMSO-d_6: δ 7.33 (C-2′, 6′, 2H, s), δ 6.89 (C-3, 1H, s), δ 6.94 (C-8, 1H, s), δ 6.46 (C-6, 1H, s), δ 5.04 (C-1″, 1H, d, *J* = 7.08Hz), δ 3.89 (C-3′, 5′- *O*-Me, 6H, s), δ 3.76 (C-6″a 1H, d, *J* =10.8Hz), δ 3.44–3.55 (C-6″b, 2H, m), δ 3.19–3.38 (C-2″, 3″, 4″, 5″, 4H, m).

^{13}C NMR 100MHz DMSO-d_6: δ182.3 (C-4), δ 164.4 (C-2), δ163.3 (C-7), δ161.4 (C-5), δ157.5 (C-9), δ148.6 (C-3′, 5′), δ140.5 (C-4′), δ120.6 (C-1′), δ105.4 (C-10), δ104.1 (C-2′, 6′), δ105.7 (C-3), δ99.7 (C-6), δ95.5 (C-8), δ100.1 (C-1″), δ77.5 (C-5″), δ76.6 (C-3″), δ73.5 (C-2″), δ70.1 (C-4″), δ60.9 (C-6″), δ56.7 (-3′, 5′- *O*-Me).

Hydrolysis of compounds **3** *and* **6**

Each compound (5.6 mg) was dissolved in 5 ml of 2 *N* HCl and then heated at 80 °C for 2.5 hr. The solution was passed through a Sep-pak C_{18} ODS cartridge (Waters), eluting with 10 ml of water and then with 10 ml of methanol to obtain equimolar quantities of D-glucose ($[\alpha]_D^{25}$ +50°, *c* 0.1, H_2O) and tricin, respectively.

Tricin*:* 400MHz DMSO-d_6: δ7.35 (C-2′, 6′, 2H, s), δ6.93(C-3, 1H, s), δ6.54 (C-8, 1H, s), δ6.20 (C-6, 1H, s), δ3.89 (C3′, 5′-O-Me, 6H, s).

^{13}C NMR 100MHz DMSO-d_6: δ181.7 (C-4), δ164.0 (C-2), δ163.6 (C-7), δ161.4 (C-5), δ157.3 (C-9), δ148.3 (C-3′, 5′), δ140.0 (C-4′), δ120.5 (C-1′), δ103.7 (C-10), δ104.7 (C-2′, 6′), δ103.6 (C-3), δ 98.8 (C-6), δ 94.1 (C-8), δ 56.4 (C-3′, 5′-O-Me).

D-Glucose*:* $[\alpha]_D^{25}$ +50°, (c 0.1, H_2O). PMR 400MHz D_2O: δ3.04–3.74 (m, β-2–6 and α-2–6), δ4.48 (d, *J* = 8.0Hz, β-1), δ5.07 (d, *J* = 3.9Hz, α-1).

Results

Probing responses to the crude rice plant extract and its separated fractions

L. striatellus frequently showed probing behavior when given the 2% crude rice plant extract plus a 15% sucrose solution. Many branched stylet sheaths (60.3±0.44 points: mean±SE) were observed on a parafilm membrane as a result of the probing behavior by the insects for 24 hr. On the other hand, when given only a 15% sucrose solution as a control, they did not leave any probing sheaths (0.0±0.0 point) on the membrane. This result clearly indicates that the crude rice plant extract contained the probing stimulant(s) for *L. striatellus.*

As shown in Fig. 2, with respect to the original activity of the crude rice extract, both BuOH (95.5±0.76 points) and ODS-40% MeOH (90.3±0.65 points) fractions revealed higher and similar probing activities for *L. striatellus* (Anova at P=0.05, DMRT). When the active ODS-40% MeOH was further separated into Fraction A and B, the bioassay results revealed that Fraction B (61.9±0.64 points) was more stimulating than Fraction A (19.2±0.49 points). Fraction B mainly

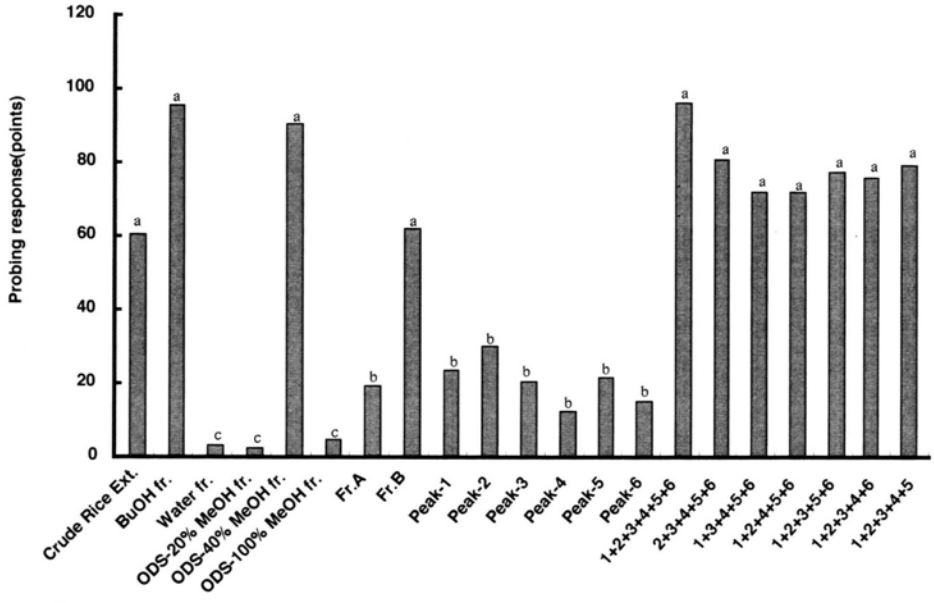


Fig. 2. Probing responses of various test solutions of rice extract fed to adult males of *L. striatellus*. Bars with the same letters are not significantly different at P= 0.05 in Anova, using the Duncann's Multiple Range Test (DMRT).

consists of six components, each of them was, therefore, isolated as a single component by using preparative HPLC and followed by bioassay. As shown in Fig. 2, separately, each of the components caused only weak responses. In contrast, when all the six components were combined, very high probing activity (96.1±0.58 points) was observed. From this results it is revealed that the bioassay for the combined compounds **1**–**6** were not significantly different from those for fraction B or the ODS-40% methanol fraction (Fig. 2). When any one of the components was excluded from these six, the resultant combination still showed high activities, which are all similar to that of the originally combined six (Fig. 2). From the above, there seems to be no key component solely responsible for the overall responses, rather a plural action of at least five components necessary for the recovery of the original probing intensity is demonstrated.

To emphasizing the probing activities by the two identified compounds **3** and **6**, the bioassay results showed that individually, compound **3** gave a probing activity of 20.2± 0.47 and compound **6** of 15.0±0.47 both of which were not significantly different from that of their combined action (29.1± 0.50 points), ANOVA, DMRT at P=0.05. As single compounds or in combination their respective activities could not recover that of the original crude rice extract (60.3±0.44 points).

There may be many minor components associated with the probing stimulation, however, judging from these bioassay results, it was concluded that **1**–**6** were the main active components in the rice plant responsible for stimulating probing activity towards *L. striatellus*.

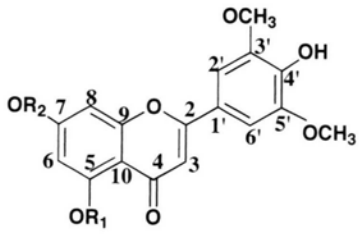
Identification of compounds **3** *and* **6**

From the positive and negative LC-MS data, the molecular weights of compounds **3** and **6** were the same and found to be 492, respectively. Their ^{1}H and ^{13}C NMR spectra also gave very similar spectra. The acid hydrolyses of compounds **3** and **6** separately led to equimolar quantities of D-glucose and tricin, respectively. Their structures were identified by direct comparison with corresponding authentic specimens described in literature (Agrawal *et al.*, 1989; Liu *et al.*, 1995; Harborne, 1999; Markham *et al.*, 1978). It was therefore concluded that both compounds **3** and **6** are *O*-glycosides with their respective glucose moieties attached to a certain hydroxyl group within the tricin aglycone.

The carbonyl carbon (177.0 ppm) shifted upfield by 4.8 ppm comparing the ^{13}C NMR spectra of **3**

and tricin. This upfield-shift (Markham *et al.*, 1978) is based on the disappearance of a hydrogen bond between the hydroxyl group at C-5 position and carbonyl carbon at C-4 position. In other words, this shift clearly shows the absence of a hydroxyl group at C-5 position, which instead, is substituted with the *O*-glucosyl. Therefore, it was concluded that a D-glucose is attached at the C-5 position. In addition to this result, since an anomeric proton of the glucose moiety shows a large coupling constant (J = 7.08 Hz) at 4.75 ppm, compound **3** could be identified as tricin 5-*O*-*β*-D-glucopyranoside, that is tricin 5-*O*-glucoside (Fig. 3).

On the other hand, in the ^{1}H and ^{13}C NMR spectra of compound **6**, both protons at H-6 and H-8 positions and carbons at C-6 and C-8 positions were shifted downfield comparing them with those of tricin. From these it was deduced that these diagnostic downshifts are due to the glycosylation of the 7-OH (Harborne, 1999; Markham *et al.*, 1978). Since the anomeric proton of the glucose moiety has also large coupling constant (J = 7.08 Hz) with a doublet at 5.05 ppm, compound **6** was identified as tricin 7-*O*-*β*-D- glucopyranoside, that is tricin 7-*O*-glucoside (Fig. 3).



Tricin $R_1 = R_2 = H$

Compound 3 R_1 = β-D-Glucopyranose, R_2 = H

Compound 6 R_1 = H, R_2 = β-D-Glucopyranose

Fig. 3. Structures of compounds **3**, **6** and Tricin.

Discussion

These two isomeric compounds were initially isolated from rice plant by Kuwatsuka (1964). Due to its widespread nature among the graminae plants it is known that tricin, the parent compound is very important as chemosystematic marker in plant taxonomy (Williams *et al.*, 1973). It is also known to be associated with rust resistance in wheat, *Triticum dicoccum* cv. 'Khapli; (Anderson, 1933) and muscle inhibitor factor (Ferguson *et al.*, 1949). As an aglycone and a glycoside both tricin and tricin 7-*O*-glucoside were respectively reported to strongly induce vir gene expression of *Agrobacterium tumifaciens* (Liu *et al.*, 1995). Tricin 5-*O*-glucoside on the other hand was recently isolated in our earlier report as one of the two compounds responsible for stimulating probing behavior of *S. furcifera* (Adjei-Afriyie *et al.*, 2000). The significance of this paper is that it is the first report on plural activity of components including tricin 5-*O*-glucoside and tricin 7-*O*-glucoside to stimulate intense probing activity towards *L. striatellus.*

It is also important to point out that from a common host plant there are apparently different compounds responsible as probing stimulants towards each of these planthopper species. We are now in the process of testing all the three planthopper species on the active components we have isolated from the rice plant. This observation is necessary for an accumulation of the facts on the probing process to determine a common strategy to manipulate the feeding behavior of these planthopper-pests. This may have the potential to develop a new pest control method for the planthoppers such as a probing inhibitor as these compounds may play the role of kairomones essential (Kim *et al.*, 1985) for directing the probing sheath of the planthopper-pests towards the sap site in the plant.

So we hope to report on the bioassay results together with the identification of the rest of the active components in due course when our studies are completed.

Acknowledgements

We are grateful to the Center for Joint Research and Development at Kochi University for measuring the NMR and LCMS spectral.

Adjei-Afriyie F., Kim C. S., Takemura M., Ishikawa M. and Horiike M. (2000), Isolation and identification of the probing stimulants in the rice plant towards the white-back planthopper, *Sogatella furcifera.* Biosci. Biotechnol. Biochem. **64**, 443–446.

Agrawal P. K., Thakur R. S. and Bansal M. C. (1989), In: Carbon-13 NMR of Flavonoids. Studies in Organic Chemistry, **39** (P. K. Agrawal, ed.). Elsevier, Amsterdam, Oxford, New York, Tokyo pp. 95–173.

Anderson J. A. (1933), Isolation of tricin as rust resistant from wheat, *Triticum dicoccum* cv. Khapli. Canad. J. Res. **9**, 80.

Auclair J. L. (1965), Feeding and nutrition of the aphid, *Acyrthosiphon pisum* (Homoptera : Aphidae), on chemically defined diets of various pH and nutrient levels. Ann. Entomol. Soc. Amer. **58**, 855–875.

Besson E., Dellamonica G., Chopin J., Kim M., Koh H. S. and Fukami H. (1985), *C*-Glycosylflavones from *Oryza sativa*. Phytochemistry **24**, 1061–1064.

Ferguson W. S., Ashworth D. B. and Perry R. A. (1949), Tricin identified as muscle inhibiting factor from the juice of lucerne, *Medicago sativa* (Leguminosae). Nature **163**, 606.

Grayer R. J., Harborne J. B., Kimmins F. K. M., Steeveson P. C. and Wijayagunsekera H. N. (1994), Phenolics in rice phloem sap as sucking deterrents to the brown planthopper, *Nilaparvata lugens.* Acta Hort. **381**, 691–694.

Harborne J. B. (1999), In: The Flavonoids. Advances in Research since 1986 (J. B.Harborne, ed.), Chapman and Hall, Boca Raton, London, New York, Washington D. C. pp. 446–447.

Kim M., Koh H. S. and Fukami H. (1985), Isolation of *C*-glucosyflavones as probing stimulants of planthoppers in the rice plant. J. Chem. Ecol. **11**, 441–452.

Kuwatsuka S. (1964), Isolation and identification of tricin glucosides. In the Study of Rice Plant Polyphenols. Nippon Nogei Kagaku Kaishi (in Japanese) **38**, 351.

Liu Y., Huang Z. H., Xiao J. G., Gu L. Q., Hu D. H., Li B. J., Wu H. M., and Ma K. (1995), The purification, identification and biological activity of metabolites capable of inducing *Agrobacterium vir* gene expression isolated from rice (*Oryza sativa* L.). Youji Huaxue (in Chinese with English summary) **15**, 72–75.

Markham K. R., Ternai B., Stanley R., Geiger H. and Mabry T. J. (1978), Carbon-13 studies of flavonoids-III. Naturally occurring flavonoid glucosides and their acylated derivatives. Tetrahedron Lett. **14**, 1389–1397.

Maxwell F. G. (1977), In: Host Relationship to Insects-Chemical Relationships. Chemical Control of Insect Behavior-Theory and Application (H. H. Shorey and J. J. Mckelvey, Jr, eds.), John Wiley & Sons Inc., New York, London, Sydney, Toronto, pp. 299–304.

Michael R. W. and Michael F. C. (1991), In: Handbook for the Identification of Leafhoppers and Planthoppers of Rice (R. W. Michael and F. C. Michael, eds.), Butler and Tanner Ltd., Frome pp. 5–6.

Mitsuhashi J. and Koyama K. (1975), Oviposition of smaller brown planthopper, *Laodelphax striatellus*, into various carbohydrate solutions through a parafilm membrane (Hemiptera: Delphacidae). Appl. Ent. Zool. **2**, 123–129.

Sakai T. and Sogawa K. (1976), Effects of nutrient compounds on sucking response of the brown planthopper, *Nilaparvata lugens* (Hemiptera:Delphacidae). Appl. Ent. Zool. **11**, 82–88.

Sogawa K. (1982), The brown planthopper: Feeding physiology and host interactions. Ann. Rev. Entomol. **27**, 49–73.

Sogawa K. (1974), Studies on the feeding of the brown planthopper, *Nilaparvata lugens* (STÅL) (Hemiptera: Delphacidae) IV, Probing stimulant. Appl. Ent. Zool. **9**, 204–213.

Williams C. A., Harborne J. B. and Clifford H. T. (1973), Negatively charged flavones and tricin as chemosystematic markers in the palmae. Phytochemistry **12**, 2417–2430.