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Influence of Active Components (Adenine) Isolated from *Leersia hexandra* Swartz on Feeding Behavior of *Nephotettix cincticeps* Uhler (Homoptera: Deltocephalidae)

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ABSTRACT

The feeding behavior of *Nephotettix cincticeps* on different adenine solutions and the estimate relative number spots of honeydew deposited on the parafilm during feeding test was investigated. The sucrose solution has a stimulating effect on the feeding of *N. cincticeps*. During feeding test (60 min) on 2% sucrose solution containing different amounts of adenine, wave form patterns of probing, salivation and ingestion were different from those obtained on 2% sucrose solution as a control. At lower concentrations of adenine (0.025, 0.05 and 0.125%), ingestion are as long and stable as a control with on average period of 53.65 min. At higher concentration of adenine (0.5 and 0.25%) ingestion period is short and disappeared. The average ingestion period was significantly reduced to 13.95 min compared to those at lower concentration of adenine ($p<0.05$). Ingestion period of *N. cincticeps* is to a large extent governed by the presence of chemical feeding stimulant (sucrose) and/or the absence of feeding deterrent (adenine). The total number spots of honeydew excreted from *N. cincticeps* on the 0.5% adenine solution was significantly decreased ($p<0.05$) than on control (2% sucrose solution) and others adenine solutions. The ingestion pattern and amount of honeydew excreted was decreased progressively with the increase concentration of adenine solutions, although the probing and salivation patterns were increased. Then, adenine has a detterent effect on *N. cincticeps*.

KEY WORDS: *Nephotettix cincticeps, Leersia hexandra,* adenine, feeding behavior, feeding deterrent.

1. INTRODUCTION

The green rice leafhopper (grh), *Nephotettix cincticeps* (Uhler), is a major insect pest of rice and in Japan and East Asian countries that causes damages through direct feeding or transmitting virus and phytoplasma pathogens (Makoto, 2015). The development and reproduction of three biotypes of the green rice leafhopper (grh), *N.cincticeps* (Uhler) were examined on resistant (unsuitable) rice varieties of Chugoku 105 (carrying the resistance gene grh1), Saikai 182 (grh2), and Aichi 80 (Grh3(t)). Biotypes 1, 2, and 3 exhibited a high survival rate, short developmental period, long adult longevity and high fecundity on the respective Grh1, Grh2, and Grh3(t)-carrying resistant (unsuitable) varieties to which they are virulent (Hirae, 2008).

There are many instances of plant secondary compounds acting as feeding stimulants, which usually inhibit or deter insect feeding. An insect feeding deterrent is defined as a chemical that inhibits feeding, although it does not kill the insect directly. Studies of naturally occurring antifeeding behavior of insect have revealed the presence of feeding deterrents in plants which affects to physiology and behavior of insect. Identified and synthesized antifeedants might be used as alternatives to insecticides for the protection of crops from noxious insect.

Kim (1975; 1985) noted that the balance between feeding stimulants and feeding deterrents which determine the plants suitability or unsuitability for the leafhopper. Liu and Takahashi (1990) evaluated the host plant suitability to three species of green leathoppers, *Nephotettix* spp., by intrinsic rate of increase (r), net reproductive rate (Ro), survival rate (S), growth index (I) and phloem feeding time (Ip) on two species of weeds and six cultivars of *Oryza sativa* L. and found the rice variety 'Nihonbare' to be a suitable plants for three species of green leafhopper. Taducan and Te-tep were found to be unsuitable plants for *N. cincticeps* Uhler and *N. nigropictus* (stall). *Leersia hexandra* Swartz is a host plant for *N. nigropictus* but not for *N. virescens* Distant. Further Liu and Takahashi (1991), tried to extract the substances responsible for the unsuitability of the plants to the leaf hoppers and to find the mechanism of unsuitability as well. They extracted the unsuitable plants with water and other organic solvents, and found that the water extract from the unsuitable plants caused high mortality to the three *Nephotettix* spp. and clearly inhibited their feeding. They concluded that the unsuitability and mortality are mostly due to a feeding deterrent effect. Based on these observations, Liu and Takahashi (1991) quantitatively measured the effect of the water extract on the insect feeding behavior. They fed *N. virescens* on sucrose solutions containing a basic or an amphoteric fraction of the water extract and analyzed the feeding patterns recorded electronically. When *N. virescens* was fed on a sucrose solution or that containing the amphoteric fraction, the insect showed a normal feeding pattern supposed to be a phloem ingestion. Fed on a sucrose solution containing the basic fraction, feeding pattern of phloem ingestion was clearly reduced. Active components which are expected to be in the basic fraction was not further analyzed.

In present studies, the author identified adenine as one of the major chemical compounds included in the basic fraction extracted from *leersia hexandra,* an unsuitable plant for leafhopper *N. cincticeps,* then tasted whether

adenine actually has feeding deterrent activity to the leafhopper. He analyzed the effect of adenine on the feeding behavior of *N. cincticeps* and proved that adenine is a substance which is responsible for the inhibition of feeding.

2. MATERIALS AND METHODS

Rearing and Maintenance of the Insect: On species of genus *Nephotettix, Nephotettix cincticeps* (Colony in Research Station, Takeda Company) was reared and maintained on Nihonbare seedlings at Pesticide Research Institute, Kyoto University. They were kept in plastic cages (30 x 28 x 25 cm) which is the same of Liu and Takahashi (1990) at temperature 25^{0}C and photoperiod of 16L-8D.

Plant Extract: *Leersia hexandra* plant (3.7 Kg) were immersed in methanol and extracted three times. The crude methanol extract was concentrated in a rotary evaporator under reduced pressure and the concentrate was separated by water and hexane. After partitioning three times with chloroform and three times with ethyl acetate, the water soluble extract was concentrated *in vacuo* and lyophilized. The water soluble materil was further fractionated into neutral, acidic, basic and amphoteric fractions on ion exchange resin column (Dowex 50 W-X8, Dowex 1-X8, 200-400 mesh).

Isolation and Identification of the Basic Fraction: The basic fraction was fractionated by ODS column chromatography and methanol as eluent with increasing concentration of methanol in water. One of the major fractions was further purified by HPLC, Shimadzu LC-10AS with detector Shimadzu SPD-10A (UV-VIS) with acetonitrile in 60 mM sodium acetate buffer (pH 6.0) as mobile phase with increasing concentration of acetonitrile as mobile phase through a column chromatography (Wakosil-PTC, 4.0 mm i.d x 200 mm) and flow rate was set to 1.0 ml/min. EI-MS was recorded on Shimadzu QP-1000Ex, ionization at 70 ev.

Preparation of Feeding Test Solutions: To determine an optimal concentration of the sugar solution, 1, 2 and 4 % sugar concentrations were used on preliminary feeding test. Two percent sugar solution maintained stable and long feeding to the insect. Test solutions contained 0.025, and 0.05, 0.125, 0.25 and 0.5% adenine in the 2% sugar. Two percent sugar solution was used as the control.

Recording of Feeding Behaviour: Electronic Measurement of Insect Feeding (EMIF) followed the method Liu and Takahashi (1991) which was carried out on an electric insect feeding recorder (Kyoto Soft Ltd., Japan) at a 500 Hz and 0.5 V alternating current source. Sensitivity was set to 4 (conductivity 0.5 mm/sec).

Recording electrode, 15 μm diameter gold wire (Tanaka Denshi Kogyo K.K, Saga, Japan), was attached to the dorsum of the green leafhopper, *N. cincticeps* with an electrically conductive paint, Dotite D-550 (Fujikura Kasei Company, Ltd., Sakaecho, Sano, Tochigi 327, Japan) and the test insect was placed on the Parafilm® stretched on the feeding chamber, which contain feeding solution. The test insect could ingest in the feeding solution through the film. The other electrode was placed in the solution so that voltage could be applied. The feeding response of the insect was recorder on a strip-chart recorder at a speed of 1.0 cm/min. activities of probing, salivation, and insertion of styles into the feeding solution were estimated by analyzing the wave forms recorded on the recorder. Feeding behavior was recorded for one hour (60 min), and 10 insect were used for each test solution. All test were conducted under a constant temperature of 25^{0}C and photoperiod of 16L-8D.

3. RESULTS AND DISCUSSION

Isolation and Identification of Adenine: The basic fraction of the water extract was separated on the ODS column chromatography. One of the major fraction was further purified on the Wakosil-PTC HPLC (Fig.1), which gave a component as a single peak on HPLC. Mass spectrometric data of the isolated component was identical to that adenine (Fig.2). Compared with the chromatographic retention time and MS of the authentic sample, adenine was identified as a component of the basic fraction. In the following experiments, it was analyzed whether adenine inhibits the feeding of *N. cincticeps*.

Effect of adenine concentration on feeding behaviour and honeydew drops on the Parafilm: The feeding response of an insect after it has alighted on a plant involves the initial feeding response, and the continued feeding response. The initial feeding response of plant hoppers is defined as probing (application of proboscis to the plant), salivation (continuation of probing) and insertion of its stylets into the food substrate (Beck, 1965). On EMIF, feeding responses are clearly identified as a single, sharp peak isolated from others. Salivation is a group of high peaks which usually follow after probing. The continued feeding response (ingestion) is distinctly identified a series of continuous strong and long frequent peaks. Most the sucking insects probe into plant tissues with their stylets, they discharge a viscous mixture of saliva, which coagulates and forms into a feeding mark (salivary flange) and salivary sheaths (Miles, 1999; Naito, 1964). It has been reported that aphids secrete watery saliva from the onset of penetration into the plant tissue (Moreno, 2011). Thus, saliva is involved from the beginning of insect-plant encounter, and is considered to contain some molecules that modulate, evade, or suppress plant defense, enabling the insect to feed on sap safely and successfully (Miles and Peng, 1999). Nevertheless, some other molecules found in saliva induce plant defense response. Therefore, the saliva may play a crucial role in determining the compatibility between the insect and the plant.

Feeding activity of *Nephotettix cincticeps*is mostly governed by the balance between chemical feeding stimulants (sucrose) and deterrents (adenine). Saxena and Khan (1985) reported that the plants sprayed with 10% neem oil showed a deterrent effect to green leathopper. The insect became restless, probed repeatedly, salivated profusely and feeding activity of insect decreased with restlessness. Our results in Fig.3, also proved that the leafhopper, *N. cincticeps* did not ingest with the increased of adenine concentration in sucrose solution. Liu and Takahashi (1990), reported that the water soluble extracts of the unsuitable plant caused higher mortalities in the green leafhopper. On unsuitable plants the green leafhopper tried to ingest, excreted and finally suffered high mortality. From this aspect, the basic materials act as toxic. However, the ingestion of the green leafhopper was also suppressed by the presence of the basic materials. This these basic materials have two effects to green leafhopper, toxic and feeding deterrent effect. Jung (1995), reported that the mortality of *N. cincticeps* nymphs were high or on resistant varieties (Norin-PL6) than on susceptible varieties (Toyonishiki). Earlier reports that (Norin-PL6) and two indica varieties (Lepe-Dumai and Taducan) exhibited an antibiosis effect on *N. cincticeps* (Kishino, 1987; Liu and Takahashi, 1990).

Feeding patterns of *Nephotettix cincticeps* on adenine solution were shown in Fig.3. *N. cincticeps* on 2% sucrose solutions showed a typical feeding behaviour on EMIF (Fig. 3-I). The sucrose solution has a stimulating effect on the feeding of *N. cincticeps*. During feeding test on 2 % sucrose solution containing different amounts of adenine, wave form patterns of probing, salivation and ingestion were different from those obtained on 2 % sucrose solution (control). At lower concentrations of adenine (0.025, 0.05, and 0.125 %; Fig. 3-II, 3-III and 3-IV), ingestions as long and stables as control, with the average period of 53.7 min (Fig. 3-I). However, probing behaviour increases with the increase of adenine concentration. At higher concentration of adenine (0.5 and 0.25 %; Fig. 3-V and 3-VI), ingestion period was short or disappeared. The average ingestion period was significantly reduced to 14.0 min ($p<0.05$). The feeding period of the *N. cincticeps* on different solutions during one hour is shown in table.1. Meanwhile the number of spots of honeydew collected from *N. cincticeps* on different solutions during one hour period are shown in Table.2. The total number of spots of honeydew excreted from *N. cicticeps* on the 0.5% adenine solution was by significantly decreased ($p<0.05$) lower compared to the control (2% sucrose solution) and others adenine solutions. Although the differences between 2% sucrose solution (control) and 0.025 adenine solutions 0.125% adenine solution and 0.05% adenine solution were statistically significant. According to the (Liu and Takahashi, 1991), the major feeding patterns of the green leafhoppers were classified as a ingestion and a non-ingestion patterns based on observation of the honeydew excretion. Du[27]g the non-ingestion pattern, no honeydew excretion was observed[11] owever, Oya and Sato, (1981) compared the feeding habits and honeydew component of the *N. cincticeps* on the resistant and susceptible varieties of rice. The leafhopper excre[11] less honeydew on resistant varieties than on susceptible varieties. The honeydew derived from the insects on the resistant varieties contained a small amount of sugars, where as that on the susceptible varieties contained a large amount of sugars composed of four kinds of sugars. Jung (1995), analyzed amino acids and sugars contained in honeydew of adults males of *N. cincticeps* on both susceptible varieties (Norin-LP6). They found that total amino acid and sugar contents in honeydew were less on Norin-LP6, whereas on the Toyonishiki contained a large amount of sugars. Furthermore, in HPLC analysis, sucrose, glucose and fructose were confirmed as the main component sugars in honeydew from the leafhoppers on Toyonishiki seedlings and none of the sugars was detected honeydew from Norin-PL6. Our results indicated that the ingestion pattern and amount of honeydew excreted was decreased with the increase of adenine concentration, while the probing and salivation patterns were increased. Then, adenine has a deterrent effect on *N. cincticeps*.

In the present study, we proved that adenine, a major component of the basic materials in *Leersia hexandra*, has feeding deterrent effect on *N. cincticeps*. This is the first study that identify a chemical component having feeding deterrent effect leafhopper. In the basic fraction of *L. hexandra,* there are other chemical components which might ha[21] feeding deterrent activity.

Table.1. Means of various events in feeding of *Nephotettix cincticeps* on the different test solutions during 60 min periods (Means ± SE)*

Solution**	Probes (No.)	Salivation (min)	Ingestion (min)
2.00% sugar	6.70 ± 1.71 a	5.90 ± 0.71 a	53.65 ± 0.80 a
Sugar + 0,025 % adenine	7.32 ± 1.00 a	5.94 ± 1.62 a	46.74 ± 1.38 a
Sugar + 0,050 % adenine	13.80 ± 20.30 ab	17.40 ± 1.43 ab	40.70 ± 1.44 ab
Sugar + 0,125 % adenine	18.40 ± 1.25 b	18.60 ± 2.98 b	36.85 ± 3.28 b
Sugar + 0,250 % adenine	42.60 ± 5.10 c	34.80 ± 1.68 c	19.55 ± 1.69 c
Sugar + 0,500 % adenine	42.90 ± 4.34 c	40.75 ± 2.83 c	13.95 ± 1.99 c

Means within a column followed by the same letter are not significantly different ($p<0.05$), Duncan's Multiple Range Test (1951).

*Average of 10 replications, each replication using a new insect and new adenine, sucrose solution; **All the adenine and sucrose solution were prepared in distilled water.

Table.2. Relative number of the spots of honeydew excreted by *Nephotettix cincticeps* on the different solutions during 60 min periods (Mean ± SE*)

Solution**	Honeydew drops per Insect (No)
2.00% sugar	19.1 ± 0.85 a
Sugar + 0,025 % adenine	16.1 ± 0.79 a
Sugar + 0,050 % adenine	11.3 ± 0.57 b
Sugar + 0,125 % adenine	10.5 ± 0.94 b
Sugar + 0,250 % adenine	5.5 ± 0.50 c
Sugar + 0,500 % adenine	3.3 ± 0.42 d

Means within a column followed by the same letter are not significantly different ($p<0.05$), Duncan's Multiple Range test (1951). *Average of 10 replications, each replication using a new insect and new adenine, sucrose solution; **All the adenine and sucrose solution were prepared in distilled water.

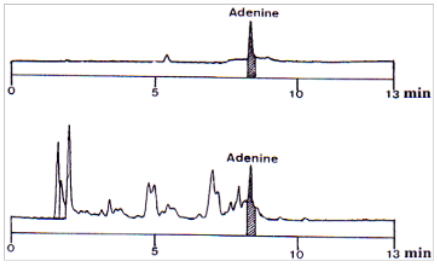

Figure.1. HPLC of authentic specimen of adenine and basic fraction from *Leersia hexandra*

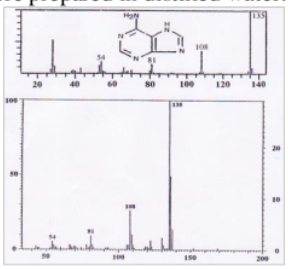

Figure.2. Mass spectrum of authentic specimen of adenine and basic fraction from *Leersia hexandra*

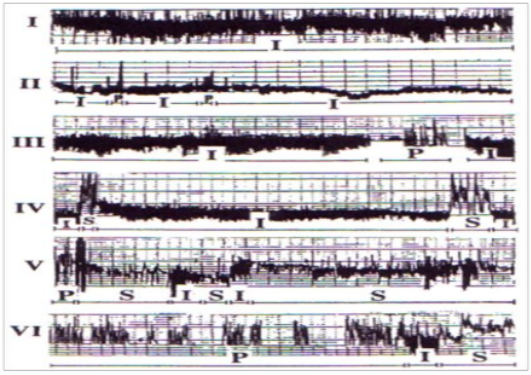

Figure.3. Feeding patterns of *Nephotettix cincticeps* on adenine solution, I: 2 % sucrose solution (control), II: added 0.025 % adenine, III: added 0.05 % adenine, IV: added 0.025 % adenine, VI: added 0.5 % adenine

P: Probe; S: Salivation; I: Ingestion

4. CONCLUSION

At lower concentrations of adenine (0.025, 0.05 and 0.125%), ingestion are as long and stable as a control with on average period of 53.65 min. At higher concentration of adenine (0.5 and 0.25%) ingestion period is short and disappeared. The average ingestion period was significantly reduced to 13.95 min compared to those at lower concentration of adenine ($p<0.05$). Ingestion period of *N. cincticeps* is to a large extent governed by the presence of chemical feeding stimulant (sucrose) and/or the absence of feeding deterrent (adenine). The total number spots of honeydew excreted from *N. cincticeps* on the 0.5% adenine solution was significantly decreased ($p<0.05$) than on control (2% sucrose solution) and others adenine solutions. The ingestion pattern and amount of honeydew excreted was decreased progressively with the increase concentration of adenine solutions, although the probing and salivation patterns were increased. Then, adenine has a different effect on *N. cincticeps*.

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