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Behavioral and Olfactory Responses of Rice Green Leaf Hopper, *Nephottetix virescens* (Distant) to Volatile Cues from Tagbak (*Alpinia elegans* (C. Presl) K. Schum)

Susan May F. Calumpang[1], **Gideon Aries S. Burgonio**[2], **Marcela M. Navasero**[1] and **Mario V. Navasero**[1]

[1]University Researchers and [2]Project Staff, National Crop Protection Center-Crop Protection Cluster, College of Agriculture, University of the Philippines Los Baños, 4031, College, Laguna, Philippines

The behavioral and olfactory responses of male and female rice green leafhopper, *Nephotettix virescens* (Distant), an important vector of tungro, was studied with a view to elucidate the mechanism for reduced green leaf hopper population in rice fields with tagbak (*Alpinia elegans* (C.Presl.) K. Schum) as practiced by farmers in Infanta, Quezon, Philippines. The effects of volatile organic chemicals released from leaf discs of tagbak were investigated using the Petri plate bioassay; and chemicals from tagbak headspace and essential oil were tested individually in Y-tube olfactometric bioassays. Our results demonstrated that *N. virescens*, regardless of sex, were apparently repelled by the odors released from the leaf discs of tagbak. The chemicals that repelled GLH were a-pinene, α –terpinene, ß -phellandrene, linalool, ß -pinene, p-cymene, camphene, 1,8 cineole, and citronellol. The mechanism of action of tagbak as an insect repellent was elucidated. The use of tagbak for insect pest management in rice production can be promoted in areas where this plant abounds to reduce dependence on synthetic insecticides. It can be a useful pest management strategy in organic and low-input rice production.

Key Words: GC-MS, insect, repellent, volatiles, Y-tube olfactometer

INTRODUCTION

Many plants have natural defenses that repel pests. Plant chemistry is a very important source of information for insects which determine its oviposition behavior and its choice of a host plant. Acceptance or rejection of a plant is determined by the overall effect of the opposing positive and negative semiochemical cues that the insect receives from the environment (Dethier 1982). The identification of plants (crops or weeds) that provide semiochemicals beneficial to crops, such as repellents for the insect pests and/or attractants to parasitoids and other natural enemies, is important for pest management in the field.

Non-host chemicals may be used for insect pest management in crops by interfering with orientation to the host plant. The influence of non-host plant volatiles on the olfactory, feeding and oviposition behavior of major insect pests and various natural enemies have been extensively studied (Schoonhoven 1968; Perrin and Philips 1978; Dethier 1982; Bell and Carde 1984; Harborne1988; Theunissen 1994). For example, onion thrips (*Thrips tabaci* Lindeman) were significantly deterred by the essential oils of marjoram (*Origanum majorana* L.), lavender (*Lavandula angustifolia* L.) and mint (*Mentha arvensis* L.), and by the oil of rosemary (*Rosmarinus officinalis* L.). Furthermore, thrips feeding damage was reduced as a result of linalool and eugenol application. Evaluation of the potential of biologically active plant volatiles against *T. tabaci* may provide a new approach to the development of integrated pest management strategies (Koschiera et al. 2002).

Among the plant genera with promising essential oils used as repellents, species belonging to *Cymbopogon,*

*Corresponding author: sue_calumpang@yahoo.com

Ocimum, and *Eucalyptus* are the most cited. Individual compounds with high repellent activity include alpha-pinene, limonene, citronellol, citronellal, camphor and thymol (Nerio et al. 2010).

The use of tagbak (*Alpinia elegans* (C.Presl.) K. Schum) pseudostems by farmers of Infanta, Quezon, Philippines to repel certain insect pests (i.e. whorl maggot, stem-borer and other flying insects) in their rice fields is a common practice. Subsequent verification field trials conducted at the Central Experiment Station of the University of the Philippines Los Baños (UPLB) in 2005 and 2006 confirmed that several insect pests of rice were repelled by tagbak. When placed every two weeks starting at two weeks after transplanting (WAT) until maturity, rice green leafhoppers were significantly the least in the tagbak treatment, at 4 to 6 WAT. More importantly, tagbak treatment did not reduce the natural enemy populations in rice. Yield in the tagbak and chemical treatment were 19% and 36% higher than that of the control, respectively (Calumpang et al. 2012). These results validated the farmer practice of using tagbak pseudostems and can be a complementary strategy for IPM in low input or organic rice production. However, the mechanism(s) of action of tagbak as a repellent of rice insect pests was not known.

A deeper understanding of the chemically-mediated behavior of insects would lead to the development of an IPM component in crop production**.** Repellents emitted by plants in the ecosystem can be utilized to reduce insect pest populations. In as much as there is farmer knowledge on the usefulness of tagbak for pest management, these are guides for chemical ecology studies to provide the basic information on the repellent volatile chemicals. The approach of this research is a novel direction using chemical ecology for pest management in rice, given indigenous pest management practices of Filipino farmers.

In the current study, we examined the influence of tagbak leaf discs on the host-finding behavior of the rice green leaf hopper (GLH), *Nephotettix virescens,* in Petri plate bioassays. Furthermore, we determined the olfactory response of GLH to the volatile chemical components (VOC) of tagbak.

MATERIALS AND METHODS

Plants and Insects

Green leafhopper (GLH), *Nephotettix virescens* - Initial stock of the insect was obtained from the Field Crops Laboratory, Crop Protection Cluster, UPLB-CA, College, Laguna. This was used in the laboratory in the bioassay of tagbak leaves and pseudostems. Another population of GLH was obtained from a private rearing laboratory and reared in the greenhouse at the National Crop Protection Center (NCPC), College of Agriculture, UPLB. These were used in succeeding bioassay tests in the laboratory from March to December 2012. GLH adults were reared on a susceptible rice variety, Taichung No. 1 (TN1) rice seedlings, planted in size- 4 clay pots, in the laboratory (oviposition phase) and the immature insects were reared in cages in the greenhouse. Tagbak grows in thickets in the vicinity of NCPC, the initial stock of which was obtained from Infanta, Quezon in 2000.

Petri plate bioassay

Three to seven day old unmated adult females and males were tested separately, using 1 adult per Petri plate. The adults were starved 3h prior to testing and each was used only once. One adult was used per replicate with 30 replicates per treatment. The bioassay was conducted in an air-conditioned room at 27-28 ^{o}C.

Three different treatments were set-up using: 1) 2.5cm leaf strip of TN1 (T-1), 2 strips, 2) 2.5 cm diameter leaf disc of tagbak (T-2), 3) 2 strips of rice placed parallel on top of 2.5 cm leaf disc of tagbak (T-3) to test repellency of tagbak volatiles to GLH when exposed to these substrates. Treatments 1 and 2 were used for the no-choice tests and treatment 3, for the two-choice tests.

TN1 leaves were obtained from 35- 45d old potted plants in the screen-house. Tagbak leaves were detached from the middle portion of a stalk carefully chosen from a standing crop at NCPC.

Fresh Petri plates were prepared by lining the bottom with 9 cm diameter filter paper (Whatman #3) which was moistened with 2 mL distilled water. Two strips of rice leaf were placed across each other upside down at the middle of the filter paper. Thirty plates were prepared in this manner. Another 30 plates were prepared in the same manner and leaf discs of tagbak were used, placed upside down at the middle of the dish. Additional 30 plates were prepared where leaf discs of tagbak were placed in the middle of the plate before placing two strips of rice leaf side by side on top of the disc. Evaluation were conducted by arranging the Petri plates equidistantly on a black plastic under dim light to avoid distraction of the GLH by light but to enable data gathering. Observations were visually recorded using a stopwatch.

GLH were released individually at the side of the Petri plate and their alighting times in the leaf discs were recorded, as well as the length of stay or duration, and frequency of alighting. Unresponsive males and females within the observation period were also recorded but not included in the statistical analysis.

Volatile Organic Chemicals Collection/GC-MS Analysis
The volatile chemicals of tagbak were collected by enclosing excised pseudostems in a Teflon bag (50L) and trapping the VOC in Tenax AG, followed by Soxhlet extraction in *n*-pentane and concentrated in a micro Kuderna Danish set-up. The volatile components were analyzed using capillary gas chromatography-mass spectrometry (GC-MS) on a Shimadzu 2010 GCMS with a 5% phenyl methyl polysiloxane column (30m x 0.25mm i.d x 0.5 um film thickness). The oven was maintained at 60°C for 3 mins, and programmed at 5°/min to 250°C and held for 5 mins. Injector temperature was maintained at 250°C. Ionization was by electron impact (70 eV), helium was the carrier gas. Tentative identifications were made by comparison of the spectra with authentic standard materials and mass spectral database (NIST107.Lib).

Y-Tube olfactometric bioassay
Bioassays of volatile organic chemicals of tagbak were conducted in an air-conditioned room (27-28°C , 52-59% relative humidity) using Y-tube olfactometer as described in Calumpang and Navasero, 2013. Charcoal filtered airstream was supplied to each arm of the olfactometer using an electric mini-pump, passing through the odor chamber with a filter paper dosed with the test chemical and the other arm had a filter paper dosed with hexane, serving as the control . The bioassay was conducted using 3-7 day old unmated adult females starved 3h prior to the experiment. The adults were used only once; one adult was used per replicate with 30 replicates per treatment.

Statistical Analysis
Data from Petri plate bioassays were analyzed using t-test. Olfactometric bioassays were analyzed using Wilcoxon Two-Sample test (SAS version 9, 2001).

RESULTS AND DISCUSSION

Responses of male and female Green Leaf Hopper to volatiles from leaf discs of tagbak and rice leaf-strips
Leaf discs, as arena for bioassay, have been used extensively in biological assay as a reliable method of providing chemical cues to pests and predators for their hosts and non-host plants (Ave et al. 1987; Jones and Coleman, 1988; Maeda 2005; Sato et al. 2007; Roessingh et al. 2007). It has been demonstrated that the odors emitted from leaf discs of non-host plants of GLH (Calumpang and Navasero 2013) are responsible for behavioral responses from individuals of both sexes in a no-choice and two-choice tests.

Male GLH spent significantly less time on tagbak than on rice ($t_{(3.55\ )}$, Fig.1(A) in treatments involving strips of

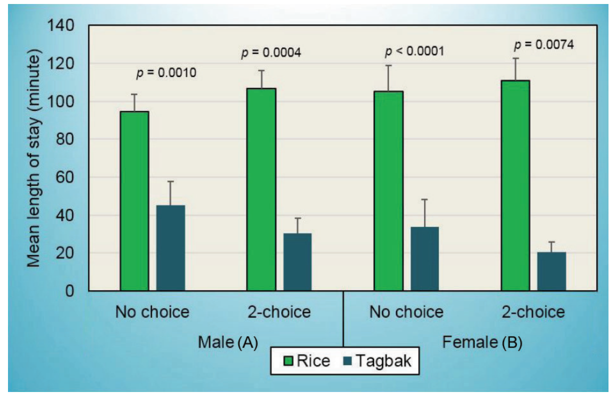

Figure 1. Mean (± SE) length of stay of male (A) and female (B) GLH, *Nephotettix virescens* on rice and tagbak in no-choice tests and 2-choice tests using leaf disks of tagbak and leaf strips of rice in Petri dish arena. Statistics: t=test; N = 30 replicates.

rice and leaf discs of tagbak offered in no-choice tests. A similar trend was observed when strips of rice and leaf discs of tagbak were offered to the male GLH in two-choice tests ($t_{(3.97)}$).

Female GLH, likewise, spent significantly less time on tagbak than on rice when rice leaf strips and leaf discs of tagbak were offered in no-choice tests ($t_{(-4.8),}$ Fig.1(B). Likewise, female GLH highly showed significant preference to rice given the choice between rice leaf-strips and leaf discs of tagbak ($t_{(5.84)}$, Fig. 1(B). The results strongly suggested that male and female GLH were repelled by the volatiles released by tagbak, as was observed when stalks of tagbak were staked out in the rice fields in Infanta, Quezon (Calumpang et al. 2012) resulting in low density of GLH. These distinct odors from leaf discs of *A. elegans* could be used as cues for host plant selection and recognition by *N. virescens.*

Male GLH showed no statistically significant difference ($t_{(-0.13)}$; Fig.3) for alighting for the first time to either rice leaf-strips and leaf discs of tagbak in no-choice tests. Similar observations were noted in male GLH when given a choice between rice leaf strips and leaf discs of tagbak in the same arena ($t_{(-0.24)}$; Fig. 2(A). For the female GLH, data showed statistically not significant alighting response to rice leaf-strips and leaf discs of tagbak in no-choice tests ($t_{(0.17);}$ Fig. 2(B) . Likewise, the female GLH, showed no significant difference in alighting response in a choice between rice leaf-strips and leaf discs of tagbak in the same arena ($t_{(-1.15)}$; Fig. 2(B).

The results suggest that the green color of rice leaf-strips and leaf discs of tagbak was innately used as cues for alighting or settling down on a host plant (rice) or even on non-host (tagbak) by GLH. However, once alighted on either rice leaf-strips or leaf discs of tagbak male or female GLH

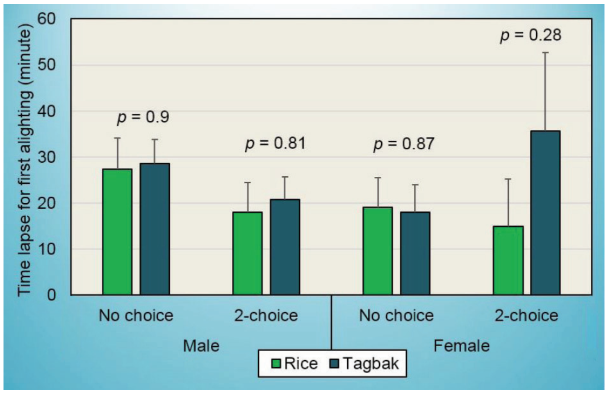

Figure 2. Mean (±SE) time lapse for first alighting of male (A) and female (B) GLH, *Nephotettix virescens* on rice and tagbak in no-choice tests and 2-choice tests using leaf discs of tagbak and leaf strips of rice in a Petri plate arena. Statistics: t-test; N = 30 replicates.

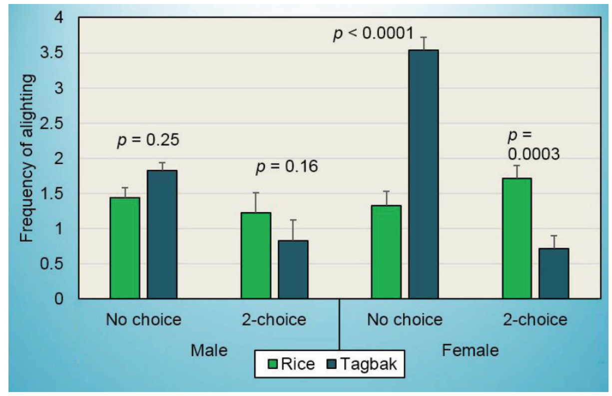

Figure 3. Mean (±SE) frequency of alighting of male and female GLH, *Nephotettix virescens* on rice and tagbak in no-choice tests and 2-choice tests using leaf discs of tagbak and leaf strips of rice in a Petri plate arena. Statistics: *t*-test; N = 30 replicates.

either fed or took off and crawled on the sides and cover of the arena before alighting again. These data suggest that additional plant stimuli were used by the GLH during host finding and or accepting behaviors. This was also observed by Backus (1985) with the *Dalbulus* leafhoppers that after contacting a plant, they can detect external plant chemicals during surface exploration and internal plant chemicals during probing and ingestion. Saxena et al. (1974) have shown that two species of *Empoasca* leafhoppers respond to olfactory plant stimuli prior to contact.

Todd et al. (1990) have shown that visual and olfactory stimuli during host finding in the leafhopper *Dalbulus maidis* made more contacts when exposed to volatile extracts from its preferred host, maize in the presence of reflected green light; a similar number of contacts when exposed to volatiles from a marginal host, gamagrass; fewer contacts when exposed to volatiles from a non-host, sorghum. Furthermore, they observed no difference between males and females in the number of contacts made with green light when exposed to maize volatiles compared to hexane alone. More contacts were made with green light than white light of similar intensity, both in the presence and in the absence of olfactory stimuli.

The male GLH showed no significant difference in the frequency of alighting in no-choice tests with rice leaf-strips and leaf discs of tagbak ($t_{(-1.18)}$; Fig. 3) as well as in two-choice tests combining the rice leaf-strips and leaf discs of tagbak in the same arena ($t_{(1.44)}$; Fig. 3).

However, in contrast to the males, female GLH made significantly more re-alightings on tagbak compared to rice in no-choice test ($t_{(-6.99)}$) but otherwise in two-choice test where the females made significantly more re-alightings on rice than on tagbak ($t_{(3.91)}$; Fig. 3). The results strongly suggest that female GLH are repelled by volatile chemicals released from the leaf discs of tagbak leading to disturbance in its orientation. This would mean reduced time on rice for feeding and eventual damage. The behavioral modification on GLH in the presence of tagbak volatiles may also result in less chance of contact between sexes for mating which may affect reproduction and population build-up of *N. virescens* in the field.

In addition, some GLH adults did not respond or remained immobile in the assays. The percentage unresponsive males increased from 10% to 23% on rice alone and on rice with tagbak, respectively (Figure 4A). On the other hand, the female response dropped from 20% to 6.7% which is consistent with the increased alighting activity when tagbak is presented together with rice. These results indicate the presence of VOCs acting as arrestant in tagbak. With tagbak VOCs inhibiting a greater proportion of males from alighting on either rice or tagbak, this would contribute to less opportunity for GLH mating in the field.

Overall, tagbak affected the movement of a total of 67% and 50% of male and female GLH, respectively in rice+tagbak treatment (Figure 4B) to include percent alighting on tagbak alone, alighting on both rice and tagbak and unresponsive. Only 33.3% and 50% of males and female GLH, respectively alighting solely on rice. The orientation of both male and female GLH was disturbed; about 30-40% made repeated alightings between rice and tagbak in choice tests indicating possible disturbance in orientation of the insects. This behavioral changes would also contribute to less time on rice for feeding and eventual damage.

Because volatiles are also present in rice, GLHs are not expected to be attracted to the volatiles from tagbak, a non-host. The results clearly show that for GLH,

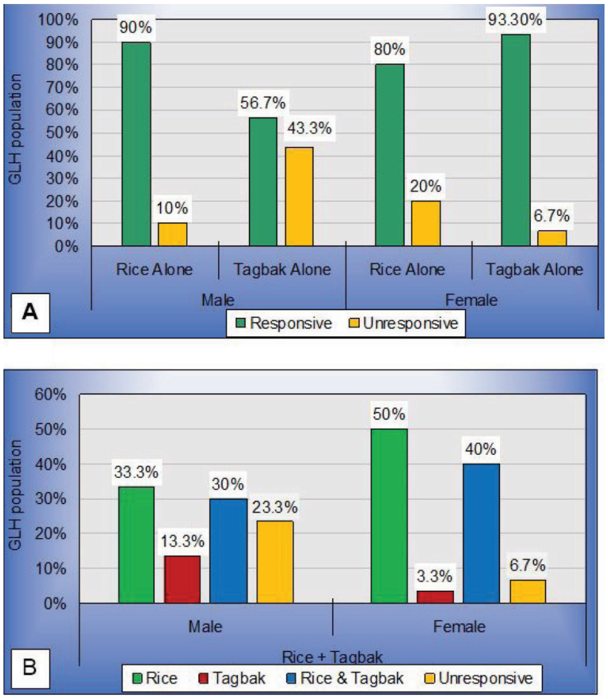

Figure 4. Mean percentage of unresponsive GLH (male & female) in tagbak and rice alone treatments (no-choice) (A) and rice+tagbak treatment (choice test) (B).

tagbak VOCs signal a non-host compound and therefore possess low acceptability. It should be noted that care must be taken when interpreting these results. It is not clear what the natural concentrations of plant odors that are experienced by GLH since concentrations near the leaf surface under field conditions can be much higher than those obtained from the leaf disc samples. However, since the sensory and behavioral responses in the laboratory bioassays are consistent with field observations, the data strongly suggest that the size of leaf discs used in the experiment represents the actual range in the field.

Olfactory response of female GLH to chemical volatiles of tagbak

GC-MS analysis of headspace chemicals showed the presence of α-pinene, β–pinene, 1,8-cineole, linalool, p-cymene, and limonene, among others. Some of these chemicals are not present in the essential oil components of tagbak collected by steam distillation (Oliveros and Bruce 1991).

GLH displayed repellency to chemicals found in tagbak essential oil; α-pinene and α-terpinene showed highly significant repellency (70%), followed by p-cymene = β-phellandrene (66.7%), linalool = β-pinene (56.7%), citronellol = 1,8 cineole = camphene (53%) and limonene (50%) (Fig. 5).

Alpha-pinene, limonene, citronellol, citronellal, camphor and thymol possess high repellent activity and have the potential to provide efficient, and safer repellents for

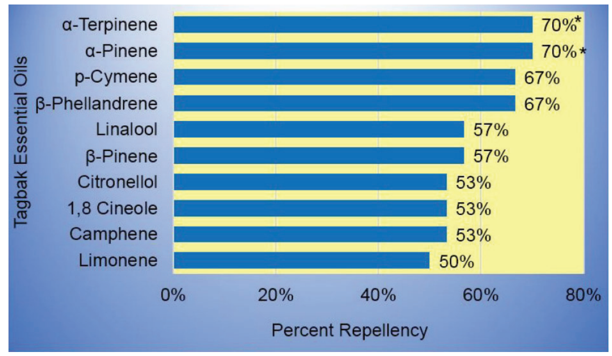

Figure 5. Response of female *Nephotettix virescens* to essential oil components of tagbak in Y-tube olfactometric bioassays. [Wilcoxon Two-Sample test (* Indicates a significant preference for that treatment (P<0.05); the absence of asterisks indicate no preference. The number of GLH adults tested was 30 for all components .)]

humans and the environment (Nerio et al. 2010). *Fraxinus pennsylvanica* volatiles proved significantly repellent to gypsy moth larval due to the combined repellencies of linalool, methyl salicylate, and farnesene (Markovic et al. 1996). In laboratory tests, ethyl acetate extracts of *Hyptis suaveolens* Poit. and *Rhododendon tomentosum* (Stokes) H. Harmaja and *Myrica gale* L. significantly reduced probing activity of *Aedes aegypti* (L.). These contained β-caryophyllene, (−)-sabinene, β-pinene, limonene, α-pinene, bergamotene. α-phellandrene, and myrcene (Jaenson et al. 2006). Other volatile chemicals such as α-terpineol, nerolidol, δ-cadinene, β-eudesmol, terpinolene, and cedrol showed high repellent activity against *Cryptomeria* bark borer (Yatagai et al. 2002).

The VOCs of tagbak are quite similar to other *Alpinia* species' essential oil content. The major components of *Alpinia pahangensis* Ridl. and *Alpinia purpurata* (Zingiberaceae) were β-pinene , *α*-pinene, β-caryophyllene and limonene (Awang et al. 2011; Santos et al. 2012). The most abundant components in the leaf and pseudostem oil of *Alpinia conchigera* Griff. included β-bisabolene, β–pinene, β-sesquiphellandrene, chavicol and β-elemene, and β-caryophyllene (Ibrahim et al. 2009). The essential oils of *A. galanga* leaves and stem are rich in 1,8-cineole, camphor , β-pinene, (*E*)-methyl cinnamate, bornyl acetate and guaiol (Jirovetz et al. 2003).

Tagbak is endemic in the Philippines and can be found in Apayao, Amburapu, Lepanto, Nueva Viscaya, Pampanga, Bulacan, Nueva Ecija, Bataan, Rizal, Laguna, Quezon, Sorsogon, Polilio, and Leyte, growing in thichets along streams and low and medium altitudes (www.bpi.da.gov.

com.ph). In these places where tagbak is abundant, rice farmers can use it in their organic or low input rice production as a pest management strategy.

CONCLUSION

The behavioral responses of *N. virescens* to the leaf discs and repellent reaction to chemicals present in headspace of *A. elegans* provide evidences for an interaction between visual and olfactory stimuli during host-finding/selection for this species of leafhopper. Volatile organic chemicals from tagbak mask the chemical cues from rice thus reducing the possibility of mating and oviposition of GLH in the field. The use of tagbak for insect pest management in rice production can be promoted in areas where this abounds to reduce dependence on synthetic insecticides. It can be a useful pest management strategy in organic and low-input rice production.

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