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Preference Assessment of White-backed Planthopper Feeding on Rice

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We are very grateful to your invitation on publishing our results on JoVE journal, and thanks again for your kindly extending the deadline of my submission.
I enclose a manuscript entitled Preference Assessment of White-backed Planthopper Feeding on Rice Using a Simple Method, which I submit for possible publication in the Journal of JoVE. All of the authors agree to the submission of this paper.

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We thank you for considering this work and look forward to your response.

Sincerely

Zhang Lei
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TITLE:

Preference Assessment of White-backed Planthopper Feeding on Rice

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KEYWORDS:

rice, resistance, nonpreference, *Sogatella furcifera*, WBPH, BPH

SUMMARY:

This study presents a simple and feasible method to assess the nonpreference resistance to white-backed planthoppers who are feeding on rice under laboratory conditions. Improvement of the strategies and makeup of the current method of identification of the resistance to white-backed and brown planthoppers are discussed.

ABSTRACT:

Exploiting insect-resistant rice germplasm resources and related genes is the primary need for breeding insect-resistant varieties, but the accuracy of the identification of insect-resistant phenotypes of rice is a major difficulty. It is urgent to develop a new method or improve existing methods to screen rice for insect resistance. This article describes a simple and feasible method to assess nonpreference-type resistance of rice to the white-backed planthopper (WBPH), *Sogatella furcifera*, in the laboratory. The preference of adult WBPHs feeding or inhabiting on maturing rice plants is continuously analyzed by pairwise comparison. The dynamic changes of WBPHs on rice plants are recorded and compared as an index of resistance identification. The current method is simply operable and easily observable and has a short cycle. The use of this method could be extended to investigate the feeding and oviposition preference of similar hemipterans, such as the brown planthopper (BPH), *Nilaparvata lugens* (Stål).

INTRODUCTION:

Rice is a staple food for over one-third of the world's population, and more than 90% of rice is produced and consumed in Asia[1,2]. The WBPH and BPH are the most destructive pests of rice and a substantial threat to rice production[3]. From the perspective of cost and environment, the breeding and application of insect-resistant rice is the most effective approach to control the damage caused by planthoppers[4–6]. Accordingly, the screening of resistant rice germplasm resources is a key prerequisite for breeding insect-resistant rice. The accuracy in the identification of rice-resistant phenotype is helpful for fine mapping and further functional research of target genes. However, phenotypic identification has become a major difficulty due to the complexity of the resistance mechanism. Rice's resistance to pests can be divided into three types, namely antibiosis, tolerance, and nonpreference[7]. Each type reflects a different aspect of the resistance mechanism of rice to pests. At present, the most widely used method of screening for resistance to planthoppers is the standard seedbox screening technique (SSST) which can be used to quickly identify the phenotypic resistance of a large number of rice plants and to obtain candidate resistant germplasm lines in a short time[8].

However, the SSST method only reflects the resistance of rice at the seedling stage and is more effective in assessing tolerance-type resistance mechanisms. Rice's resistance to insects is also reflected in antibioses, such as nymph survival rate, nymph duration, and egg hatching rate, and in nonpreference, such as habitat, feeding, and oviposition preference[9]. In addition, the performance of rice seedlings for resistance is often not very stable. With the growth of plants, resistance tends to become more stable. Therefore, the SSST method cannot completely reflect the resistance level of rice. Moreover, rice's resistance to pests varies at different growth stages, and there are obvious differences in resistance mechanisms between seedling and maturing plants. Studies have shown that maturing rice plants can release volatile secondary metabolites to avoid infestation by insect pests, which are manifested by the insect's nonselectivity in feeding or oviposition on the rice plant[10–11]. This is also a very critical kind of resistance mechanism, which plays an important role in preventing insect pests and ensuring rice yield at maturity.

At present, the identification of rice's resistance by nonpreference is still a challenge. In this case, two main approaches are currently used. On the one hand, planthoppers and rice plants are put in a square nylon net cage[12]. Although this approach is considered to be relatively efficient for carrying out experiments on multiple rice lines simultaneously, it requires a larger experimental space and, thus, causes some difficulties in observation and counting due to nontransparent nylon net materials. On the other hand, the Y-tube olfactometer method is used in insect selection experiments according to the difference in volatile substances released from rice. This method facilitates easy observation because of its glass container[14]. One of the major limiting factors of this method is that it can only judge volatile smell, and it also has a strict requirement on the tightness of the experimental devices and takes a long time.

Herein, we describe an improved method for evaluating the nonpreference-type resistance of the rice plant to WBPHs, which is simple to operate and easy for observation. This method can also be used to study the habitat, feeding, and oviposition preference behavior of BPHs and other hemipterous pests.

PROTOCOL:

1. Preparation of planthoppers, rice plants, and the polyvinyl chloride cage

1.1. Planthoppers

1.1.1. Rear WBPHs on tillers of a susceptible rice variety called Taichung Native 1 (TN1) in insect-proof cages and let them reproduce naturally for generations. Choose long-winged, newly emerged female adults for further experiments.

NOTE: WBPHs were provided by the Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences.

1.2. Rice plants

1.2.1. Soak seeds of each rice line in water and put them into a climate-controlled room with parameters set to 28 °C, 75%–80% relative humidity (RH), and cycles of 14 h light/10 h dark for 2 days until germination.

1.2.2. Sow 30 germinated seeds of each tested rice line evenly in a plastic seedbox (20 cm [length] x 15 cm [width] x 10 cm [height]) which is filled with paddy soil to a depth of 3–4 cm.

1.2.3. Cover the seeds with a thin layer of fine dry soil; then, wet the dry soil with water.

1.2.4. Put the seedbox in a 200 mesh insect-proof cage (75 cm [length] x 75 cm [width] x 75 cm [height]) at 28 °C, with 75%–80% RH and a 14 h light/10 h dark cycle treatment in a climate-controlled room. Water every day to keep the soil moist. Continue growing the plants for 7 days, till they have reached the two- to three-leaf stage.

1.2.5. Choose 20 seedlings with similar growth potential, transplant the seedlings into 10 cm-diameter plastic seed pots (one seedling per pot) with a hole at the bottom.

1.2.6. Place the pots in a 200 mesh insect-proof cage (75 cm [length] x 75 cm [width] x 75 cm [height]) at 28 °C, with 75%–80% RH and a 14 h light/10 h dark cycle treatment in a climate-controlled room, with water at the bottom of the tray, for about 30 days of growing till they reach the tillering stage with one or two tillers.

1.2.7. Trim the rice plants to one tiller 48 h before starting the experiment.

1.3. Cylindrical polyvinyl chloride cage

1.3.1. Obtain transparent polyvinyl chloride (PVC) with dimensions of 120 cm x 90 cm and a thickness of 0.5 mm.

1.3.2. Make it into a cylindrical structure with a height of 90 cm and a diameter of 35 cm.

1.3.3. Use a stapler to fix the overlap area at both ends of the cylinder. Ensure the overlap area is about 90 cm in length and 10 cm in width.

1.3.4. Seal the entire overlap area from the periphery of the cylinder with pressure-sensitive tape.

NOTE: Ensure that the cylindrical cage can be placed vertical to the ground and there is no obvious gap between the cage and the ground.

1.3.5. Cut 200 mesh nylon nets, each with dimensions of 50 cm x 50 cm; prepare enough for the subsequent steps.

1.3.6. Get adequate rubber bands; make sure the diameter is about 1.5 mm and the circumference is at least 32 cm when the band is contracted.

2. Insect and rice treatment

2.1. Place a round plastic tray with a diameter of 28 cm and a height of 10 cm on flat concrete ground in a climate-controlled room with parameter settings as described in step 1.2.6.

NOTE: If the greenhouse floor is soil, find an as flat as possible surface to ensure that the tray is laid flat.

2.2. Choose two pots of different rice lines (from step 1.2.7) and put them into the tray, side by side, and fill the plastic tray with enough water.

2.3. Cover the two test rice pots with the cylindrical cage made in step 1.3.4.

2.4. Put one piece of nylon net (from step 1.3.5) on top of the cage.

NOTE: The two rice pots in the cage can be used as a group; repeat 15 sets of each group. Place the rice pots randomly in position and direction, but try to ensure that the leaves of the two rice plants do not touch.

2.5. Use a handmade suction trap to collect 40 newly emerged female WBPH adults (refer to section 1.1).

2.6. Put the WBPH adults into a glass tube (with a diameter of 2 cm and a height of 15 cm) and cover it with a sponge stopper.

2.7. Lift up a corner of the nylon net (see step 2.4).

2.8. Remove the sponge stopper from the glass tube and put the tube in the middle part of the cage to release all WBPHs.

2.9. Cover the nylon net quickly and use a rubber band to seal it to prevent the WBPHs from escaping (**Figure 1**).

3. Recording and observation

3.1. Observe the distribution of WBPHs on each rice plant at 3, 6, 24, 48, 72, 96, and 120 h after the infestation.

3.2. Record the number of WBPHs on different rice plants including leaf sheath and leaf from all directions through the transparent cage.

NOTE: Be gentle during the observation process so as not to disturb the WBPHs.

REPRESENTATIVE RESULTS:

There were three test rice lines used in this study. Rice line FY01 is WBPH susceptible and used as control group. Rice line HZ08 and HZ06 were transgenic lines in which the potential WBPH resistant X1 gene and X5 gene were introduced, respectively, based on the background of FY01. Therefore, a rice resistance comparison between HZ08/HZ06 and FY01 could reveal whether the corresponding inserted gene had a potential resistance function. In this study, the resistance of two rice plants in terms of feeding or inhabiting was compared, thus revealing whether these two genes were potentially involved in the mechanism of nonpreference-type resistance.

The number of WBPHs on experimental rice plants at different time points was recorded. The ratio of WBPHs was obtained by dividing the number of WBPHs on each plant by the total number of WBPHs on the two rice plants in the cage, excluding the WBPHs located elsewhere, such as on the ground and cage walls. A commercial software was used for further statistical data analysis. One-way analysis of variance (ANOVA) was applied, followed by Tukey's multiple range test, to assess the significant difference between the compared groups. Percentage data were transformed to arcsine square root for ANOVA. The results showed that for the comparison experiment between rice HZ08 and FY01, the number of WBPHs on both rice lines were relatively small at the initial stage from 0 to 6 h (**Figure 2A**). Most of the WBPHs did not show feeding activities and might have been in an adaptive stage. However, both the number and the ratio of WBPHs on the FY01 line were significantly different from those on the HZ08 line (**Figure 2A** and **Figure 3A**). After 24 h, the number of WBPHs on the FY01 line increased gradually. Nevertheless, the number of WBPHs on the HZ08 line remained low from the beginning to the end of the experiment after 120 h (**Figure 2A**), and the ratio showed the same result (**Figure 3A**), which was indicating an obvious preference for inhabiting and feeding on FY01. This also implied that the corresponding inserted X1 gene is a candidate gene for rice resistance and may be associated with the nonpreference resistance mechanism.

The results for the rice lines HZ06 and FY01 were different than the results for HZ08. The number of WBPHs inhabiting the rice lines HZ06 and FY01 were relatively large at the beginning, but there was no obvious difference between the number and ratio of WBPHs on line HZ06 and those on line FY01 (**Figure 2B** and **Figure 3B**). As the experiment went on, the number and ratio of WBPHs on each rice line changed slightly, but the differences were not significant. This result showed that WBPHs had no feeding or inhabiting preference between the two rice lines, and also indirectly indicated that the inserted gene X5 did not participate in the mechanism of nonpreference resistance.

These results indicated that this method could be used for the identification of rice's resistance to WBPHs. Rice line HZ08 was successfully identified as resulting in a nonpreference by WBPHs for feeding or inhabiting when compared to the control line FY01, thus showing a certain resistance to WBPHs. On the other hand, compared with the control group, rice line HZ06 showed no nonpreference resistance to WBPHs.

FIGURE LEGENDS:

Figure 1: Diagram of the nonpreference experiment of WBPHs feeding on rice in this study.

Figure 2: Dynamic changes of WBPH populations on experimental rice lines. (**A**) WBPH populations on single seedlings of HZ08 and FY01. (**B**) WBPH populations on single seedlings of HZ06 and FY01. Error bars, mean ± standard error (SE), by Tukey's multiple range test (n = 15, **P < 0.01).

Figure 3: Dynamic changes of WBPH on different experimental rice lines. (**A**) Ratio of WBPHs on single rice plants of HZ08 and FY01. (**B**) Ratio of WBPHs on single rice plants of HZ06 and FY01. Error bars, means ± SE, by Tukey's multiple range test (n = 15, **P < 0.01).

DISCUSSION:

Maturing rice plants release volatile secondary metabolites to control insect pests or reduce the mating capacity of these pests (such as in WBPHs) via a special physical structure on the leaf sheath surface, which is a key resistance mechanism[13]. In rice plants, the nonpreference is not only related to feeding but also associated with habitat and mating. However, current studies have focused on the nonpreference of nymphs[4,12], and a new method needs to be developed for the identification of adult nonpreference. The present study mainly described a method to identify nonpreference-type resistance of the maturing rice plant to adult WBPHs. The method requires a few experimental materials and the experimental procedures are simple. However, the preliminary preparation is very important since a large amount of WBPHs are needed during the experiment. To ensure the accuracy and persuasiveness of the experimental results, it is necessary to use WBPHs at similar growing stages. Considering the long growth duration of rice, it is necessary to plan the experiment well in advance to ensure that there is a sufficient source of planthoppers when the rice plants are ready. In addition, several strains of each rice line should be planted as backup. It must be noted that when performing step 2.8, do not release WBPH on the rice plant, but try to ensure that the planthoppers are evenly distributed in the cage at the beginning.

This method is more suitable for the nonpreference-type resistance identification of flying pests, especially for those insects whose feeding or habitat behavior is attracted by volatile emissions. Therefore, it is not suitable for the study of nymphs or short-winged morphs, who cannot fly. The cylindrical cage in this experiment is made up of PVC, and the physical structure will be unstable if the cage diameter is enlarged as PVC material is soft. Therefore, the current cage size is more suitable for the comparison of two rice pots at the same time, and it is difficult to accommodate three or more rice plants in one experimental group. In addition, the method is only suitable for the study of rice plants with a single tiller or two tillers, which excludes matured rice plants since they have many tillers which occupy a large volume and will be inconvenient for insect pest counting. Moreover, the observation time is limited to a certain extent because the insects cannot be observed at night.

A traditional raising container for planthopper experiments is usually made up of a 200 mesh nylon net cage with zippers, usually large in size[14]. Due to the low transparency of nylon net material, it is necessary to unzip the zipper when observing the insects, and they can usually only be seen from one direction. The method described in this study uses transparent PVC as the experimental container, which has the advantage of omnidirectional observation without disturbing the pests. Nylon net is used to cover the cylindrical cage, which can make up for the shortcomings of PVC's impermeability. At the same time, high transparent PVC material ensures that the rice plants and pests receive sufficient light, which is very similar to natural conditions. In addition, this method effectively reduces the space needed for the experiment and facilitates more than ten groups of experiments at the same time. By using this method, the observation of oviposition preference of female WBPHs on maturing rice plants is also possible, which could be considered as another resistance evaluation index. Overall, the method provides the basis for further effective behavioral studies of various pests, such as the population competition relationship between WBPHs and BPHs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

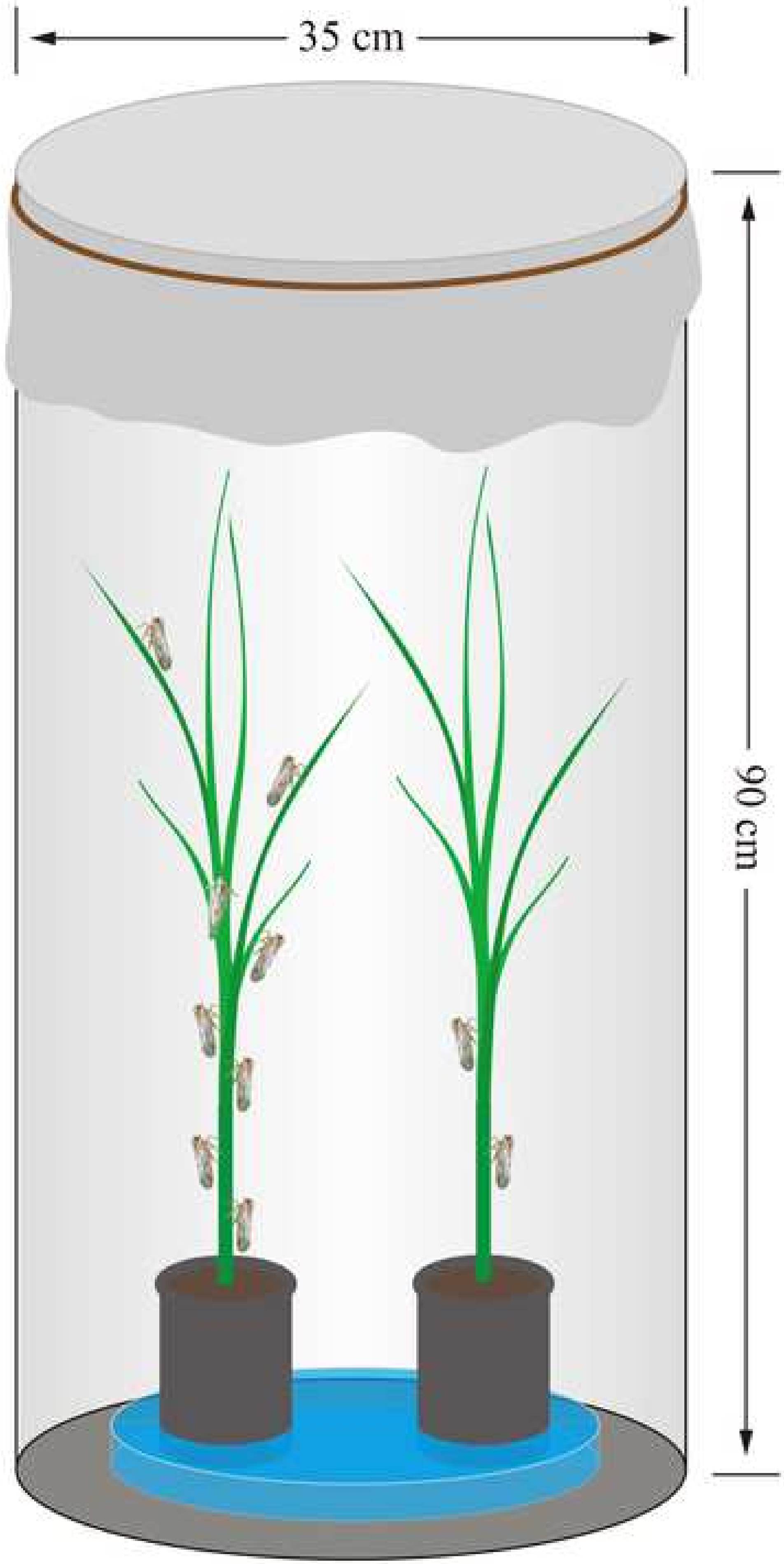


Figure 2 Click here to access/download;Figure;Figure 2.jpg

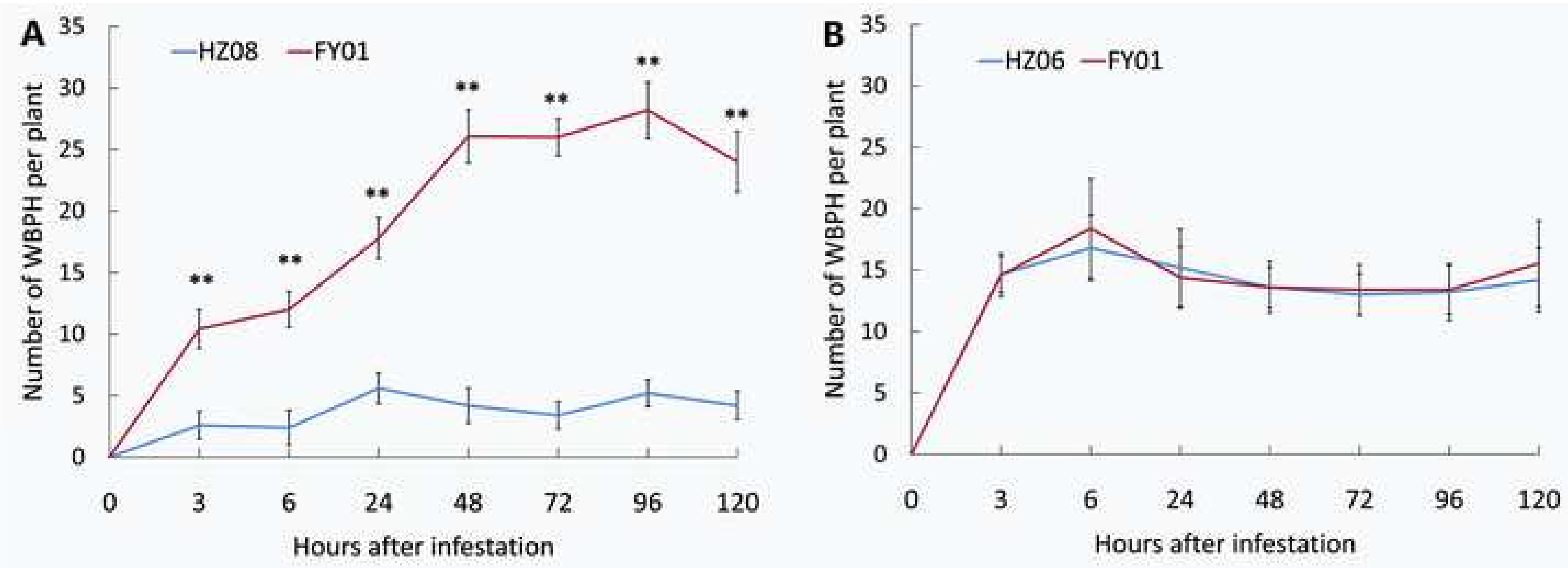
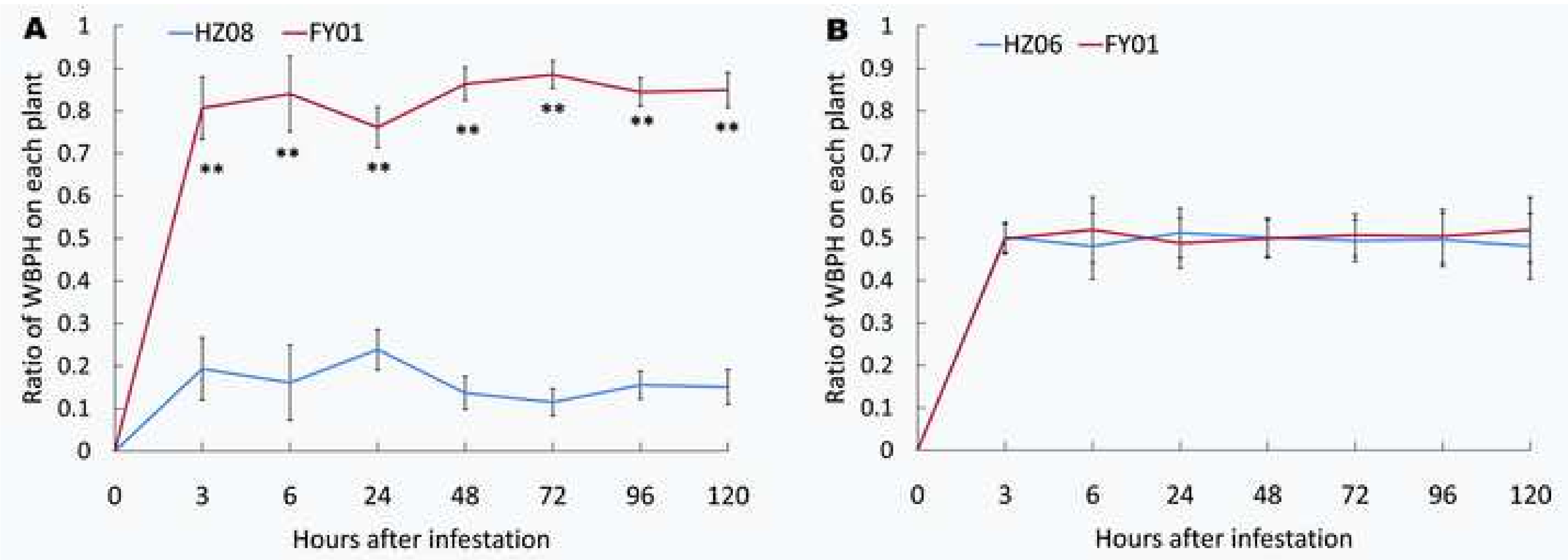


Figure 3


A
HZ08
FY01
Ratio of WBPH on each plant
Hours after infestation
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
3
6
24
48
72
96
120
**
B
HZ06
FY01
Ratio of WBPH on each plant
Hours after infestation

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
climate-controlled room	Ningbo Jiangnan Instrument	SZJYS2013	temperature, relative humidity, photoperiod contr
glass tube with sponge stopper	/	/	diameter 2 cm and height 15 cm
handmade suction trap	/	/	/
insect-proof cage	/	/	200-mesh, (L × W × H, 75 × 75 × 75 cm)
Nylon net	/	/	200 mesh
paddy soil	/	/	/
plastic seed box	/	/	(L × W × H, 20 × 15 × 10 cm)
plastic seed pot	/	/	10-cm-diameter
plastic tray	/	/	(D × H, 28 × 10 cm)
rice seed of FY01 line	/	/	60 seeds
rice seed of HZ06 line	/	/	30 seeds
rice seed of HZ08 line	/	/	30 seeds
rice seed of TN1 variety	/	/	many
Rubber band	/	/	diameter is 1.5 mm, and the circumference is 32 cn
scotch tape	/	/	/
SPSS Statistics 19.0	IBM Corporation	/	statistical data analysis
stapler	/	/	/
transparent PVC	/	/	120 cm × 90 cm dimensions and thickness of 0.5 mı

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Dear Editor and Reviewers,

Thank you for your professional comments on my manuscript. I have made a systematic revision to my manuscript in accordance with your requirements. See the details in submitted revised manuscript with tracking changes. The following words in blue are my answer to specific questions. I hope I can satisfy you.

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have carefully revised the whole manuscript, please see details in revised manuscript.

2. Please revise lines 156-159 to avoid previously published text.

We have changed that part in our revised manuscript.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We have already revised protocol steps according to your requirements.

4. Lines 87-90: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “NOTE”.

Already done, please check.

5. Line 93: Are the seeds soaked under light or dark conditions? What is the temperature?

We add the conditions for that protocol.

6. Line 94: How many seeds are seeded? 30 or 60?

We have made it clear in our revised manuscript, seed 30 for each rice line.

7. Line 99: How many seedlings are transplanted?

We have defined the number in step 1.2.5.

8. Figure 2: It is unclear how the y-axis values are obtained. Please describe what the ratio stands for. Both panels A and B contain the data for FY01, but it is confusing why the values are different in the two panels (red lines). Please explain.

We explained how we obtained the y-axis values in the RESULTS section. There are two test rice lines (HZ08/HZ06) both compared with FY01, but two groups of experiments were carried out separately, so the data for FY01 are different.

9. Lines 159-164: Where are the data (numbers of WBPH) describe here?

We add the data of WBPH number in Figure 2, and the previous Figure 2 is changed to Figure 3.

10. Lines 170-171: Figure 2B shows the changes of the ratio, not the numbers. Please include the data for numbers of WBPH.

We add the data of WBPH number in Figure 2.

11. Table of Materials: Please provide the company and catalog number for the material/equipment used. Please sort the items in alphabetical order according to the name of material/equipment.

We have improved the relevant information of the climate controlled room. However, some of the other materials or equipment are common and accessible materials, and there is no professional company or catalogue number.

Reviewer #1:

Manuscript Summary:

1. I do not think the author revised the MS carefully, the obvious error "dipterans pest" were still existed. WBPH was hemipterous pest.

We apologize for our low-level mistakes. We have revised that in our revised manuscript.

2. This method has been reported, so I still could not get the innovative aspects of this methods. Just like this paper, "Influence of water-stressed rice on feeding behavior of brown planthopper, Nilaparvata lugens (St?l)". They have used this method to investigate the behavior of BPH.

Thanks for your comments, our study mainly described a simply method to identify rice resistant varieties. In our manuscript, the protocols of this method are described in detail. Although there are some similarities with the literature you mentioned. There are obvious differences in size (d×h=20×45 cm VS 35×90 cm), and also the growth stages of rice studied are different. We still insist that our method has some innovative aspects in assessing non-preference type resistance in rice.

Reviewer #2:

Manuscript Summary:

This manuscript has been improved, i recommend to accept

Thank you for your support.

Reviewer #3:

Manuscript Summary:

This manuscript describes a relatively simple and straightforward two choice preference assay for the White-backed planthopper and demonstrate its effectiveness using two transgenic rice lines differing in the expression of different potential HPR genes (unidentified). They show that the WBPH is deterred from establishing on one of those transgenic lines, but not the other, relative to the non-transformed control. As such it would appear to be an effective assay to assess non-preference type resistance in rice so should be useful to other researchers and would presumably work just as well with other highly mobile pests of rice.

Major Concerns:

The manuscript could do with a bit of work as the English is poor and some of the sentences very repetitive.

We have made a systematic revision to my previous manuscript, and try to avoid any mistakes in grammar or any vague expression. Please check that in our revised manuscript.

Minor Concerns:

Line 37 - should provide the scientific name for BPH at first mention.

Already done.

Line 37 & Line 81 - WPBH and BPH are hemipterans - not dipterans

Already changed.

Line 49 - delete 'such as'

Already done.

Line 55-56 - sentence confusing - maybe - 'and is more effective in assessing tolerance type resistance mechanisms'

Already changed in accordance with your suggestion.

Line 60 - is reference 10 the correct reference - -would seem to have nothing to do with plant HPR but about an animal virus??

Thanks for your reminding. We have deleted reference 10 in our revised manuscript.

Line 98 and elsewhere - these appear to be glasshouses with presumably natural lighting so defining an exact day night cycle is probably not suitable - should say 'natural or ambient lighting for approximately 14h per day' ?? If they are growth cabinets with artificial lighting then more detail should be given about the strength and duration of lighting, humidity and temperature control etc

Our experiment was completed in a climate-controlled room. We have improved the information of detailed parameters in relevant places.

Line 104 - '...one tiller 48h before starting the experiment'

Already done.

Line 121 - most glasshouses have concrete floors rather than soil - so they should maybe provide an option to address that? Maybe plant the two small pots into a larger pot of soil and surround that with the PVC Sleeve? Could then water that from the base (see next point)?

We have changed that part in step 2.1.

Line 126 '..don't touch' rather than 'uncrossed', also as the experiment can go on for five days they should mention how they water them.

We change the protocol 2.1 in our revised manuscript, we put a plastic tray at the bottom of the rice pots, we are sure that they can supply enough water for two rice plants.

Line 194 - 'spawning' is usually associated with fish and amphibians rather than insects so maybe 'mating' would be more appropriate

Already changed in accordance with your suggestion.

Line 242 '...Sciences USA'

Already changed.

References - gene names and species should all be in italics, reference 10 is incorrect

We have checked through the reference part and changed that.

Table of Materials

Although not that critical - no company names or catalog numbers are mentioned

Most of the materials are common materials, and there is no professional company or catalogue number.

A climate controlled room is mentioned so this should have manufacturer details?

We have add information for climate-controlled room. Thanks again for your professional comments.