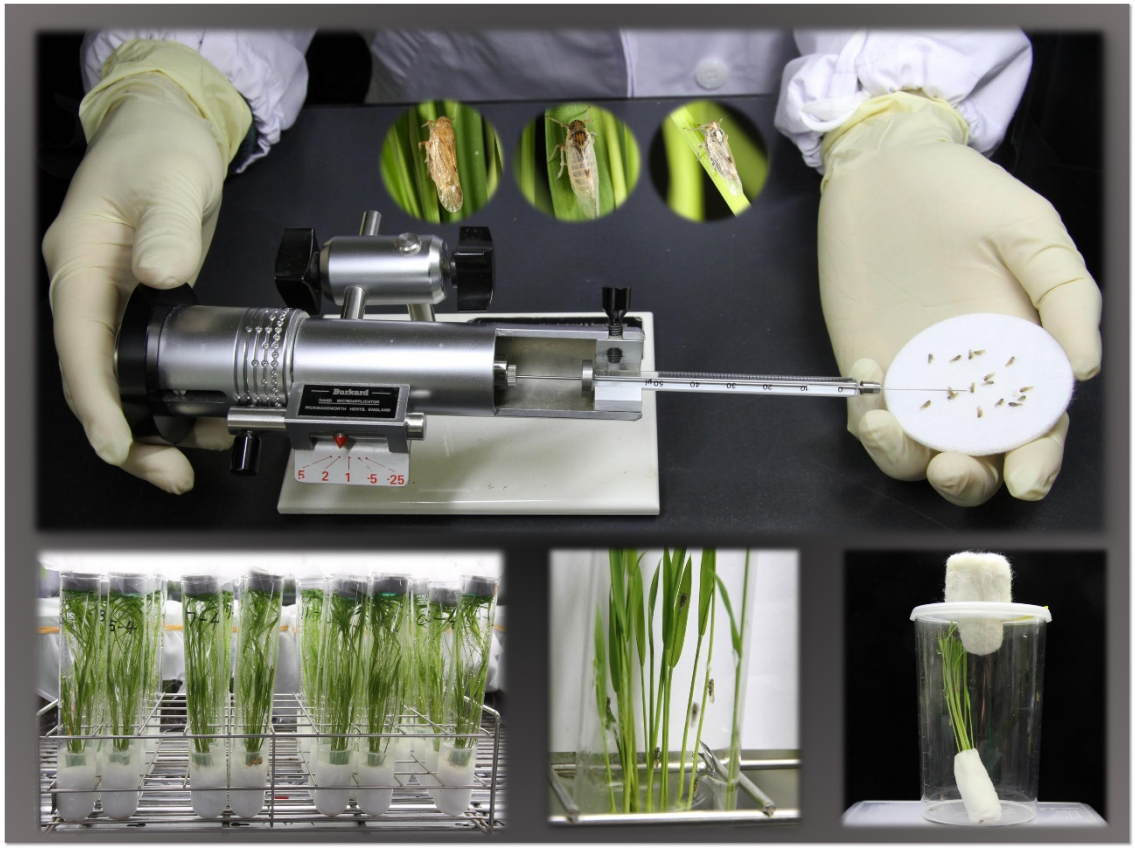
Manual for testing insecticide susceptibility in rice planthoppers



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1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål), and the whitebacked planthopper (WBPH), *Sogatella furcifera* (Horváth), are distributed widely in temperate and tropical areas of Asia. Neither of these species is able to overwinter successfully in temperate areas such as Japan because rice (*Oryza sativa* L., the only host plant) is not cultivated there in winter. The northern limit of breeding area for these species is around northern Vietnam and the most southern regions of China. Colonization by these species is therefore annual, following long-distance migration from overwintering areas in the rainy season. After the harvesting of rice in autumn, BPH and WBPH are not able to overwinter in Japan. Return migration from Japan to southern China and northern Vietnam seldom occurs because of the absence of southwest wind, except for typhoons, in autumn.

In contrast to BPH and WBPH, the small brown planthopper (SBPH), *Laodelphax striatellus* (Fállen), is able to use several plants such as poaceous crops and biennial poaceous weeds as host plants. SBPH is able to overwinter in temperate areas as diapause nymphs. SBPH is distributed widely in Japan and is observed even in Hokkaido. Although long-distance migration seldom occurred in SBPH until the mid–2000s, long-distance mass migration is currently observed from China to Japan and/or Korea in early June when China is in the wheat-harvesting season.

In general, the development of insecticide resistance in insect pests occurs where the same insecticide is used in the same place continuously. In migratory pests such as the rice planthoppers, however, insecticide resistance develops by the overuse of insecticide in the source areas, and resistant insects migrate into Japan. For such insect pest species, a continuous monitoring of insecticide susceptibility is required for forecasting these pests in the source areas such as Vietnam, and/or in Japan. Because insecticide susceptibility is not monitored continuously in the source areas, the immigrant populations of planthoppers are required to be monitored in Japan.

There are two methods for monitoring insecticide susceptibility of rice planthoppers. One method is to calculate the median lethal dose (LD_{50}) or the median effective dose (ED_{50}) and the other is the method to calculate the median lethal concentration (LC_{50}) or the median effective concentration (EC_{50}). The former method using LD_{50} or the ED_{50}–values can compare susceptibility data obtained in different years and in different places, but the latter method with the LC_{50} or the EC_{50}–values can only compare the data tested simultaneously. Thus, the methods that can calculate the LD_{50} or the ED_{50}–values are strongly recommended to observe the change of insecticide susceptibility in rice planthoppers.

In this manual, the methods to calculate the LD_{50} or the ED_{50}–values for testing insecticide susceptibility of rice planthoppers are described. In addition, the collecting and rearing methods of rice planthoppers are also described.

2. Methods for monitoring insecticide susceptibility of rice planthoppers

2.1 Leaf dipping method

In this method, rice seedlings or rice leaves are dipped completely in test insecticide solutions of several concentrations. After dipping, the seedlings or leaf are allowed to dry. This method is easy to conduct and used widely, but can only calculate the LC_{50}–value and is difficult to compare susceptibility data obtained in different years and different places using this method. For this reason, a topical application method is commonly used as a standard for testing insecticide susceptibility of rice planthoppers. The dipping method should be used only for monitoring susceptibility of insecticides such as insect growth regulator (IGR), which are not amenable for testing by the topical application method.

2.2 Topical application method

In this method, a topical applicator (e.g., hand micro-applicator produced by the Burkard Manufacturing Co., Ltd., UK) equipped with a micro-syringe is most commonly used. This method can calculate the LD_{50}–value. A simple and low cost equipment method using a repeating dispenser has also been developed (Sanada-Morimura and Matsumura, 2011). However, the topical application method is not applicable for calculation of LD_{50}–values in IGR insecticides (e.g. buprofezin) and pymetrozine which primarily control the feeding behavior.

2.3 Method for monitoring the susceptibility of pymetrozine

Because pymetrozine prevents feeding and reproduction in insects, the lethal effect by pymetrozine is not very high in rice planthoppers. Thus, the topical application method which can calculate LD_{50}–value is not applicable for monitoring the susceptibility of pymetrozine. A new method for monitoring the susceptibility of rice planthoppers to pymetrozine with ED_{50}–values has therefore been developed by combining topical application and measurement of offspring number.

3. Collection and rearing of test insects

3.1. Collection of test insects

In temperate regions, such as Japan, it is better to collect the insects in immigrant generation for BPH and WBPH. In BPH, collecting the insects in reproductive generation is acceptable if population density of immigrant generation is very low in paddy fields. At least fifty females per population are collected as test insects. It is better to collect the insects at adult stage in the reproductive generation and they should be collected from wide areas in paddy fields. Insect sampling is done from paddy fields with no insecticide application. Insects can be collected by a sweep net (Fig. 1) or from the bottom part of rice plants using a white tray and a mouth aspirator (Fig. 2). Some planthopper individuals collected from paddy fields are parasitized by a drynid wasp (Fig. 3) or by the Strepsipteran's natural enemies. Parasitized individuals are completely removed in the laboratory after insect collection. Contamination by predators of planthoppers such as spiders and the mirid bug (Fig. 4) should be avoided in planthopper samples as these predators can cause a reduction in population growth rate in laboratory rearing.

Fig. 1. Collection by a sweep net.

Fig. 2. Collection by a white tray using a mouth aspirator.

Fig. 3. A BPH adult parasitized by a larva of drynid wasp.

Fig. 4. Mirid bug (*Cyrtorhinus lividipennis*), an egg predator of planthoppers.

3.2 Identification of planthopper species

There are three species of rice planthoppers, BPH, WBPH, and SBPH. Collected insects are identified and are sorted out carefully without contamination. See Fig. 5 for general points of identification of the three planthoppers. The following criteria can be used for identification of the different species:

1) BPH: Adult body is slightly shiny and brown to blackish brown in color. Wing color is slightly brownish.
2) WBPH: Adult has a white band on its thorax in males and females. Nymphal body color is whitish.
3) SBPH: Body color of female adult is as brownish as that of BPH, but body size is smaller than BPH and is not shiny.

* See Shepard et al. (1995) for more detailed identification criteria.

Fig. 5. Female adult (left), male adult (center), and nymph (right) in the brown planthopper (BPH) (upper), the whitebacked planthopper (WBPH) (middle), and the small brown planthopper (SBPH) (lower).

3.3 Successive mass-rearing method

Several testing insects are required to test for insecticide susceptibility. For example, about 250 individuals of long-winged adult females are required for testing susceptibility for one insecticide. Thus, collected planthopper populations are multiplied by a successive rearing in the laboratory. A high-density rearing at the nymphal stage will efficiently produce abundant long-winged test insects (Fig. 6).

Fig. 6. The SBPH nymphs reared at high density.

3.3.1 Rearing environment

Rearing is carried out in a temperature-controlled room or an incubator maintained at a day length of 16L8D and a temperature of 25 ℃. It is desirable to control the relative humidity to 70–80% R.H. In case of excessive moisture condition, a dehumidification machine can be used to decrease the humidity.

3.3.2 Rearing equipment and preparation of rice seedlings

For mass-raring of planthoppers, a transparent acrylic cage (Fig. 7) containing rice seedlings is used. Soak rice seeds (belonging to a susceptible variety against rice planthoppers) in water for 3–4 days for germination. Germinated seeds can be stored in a refrigerator at 5 ℃ for 2–3 weeks. Hydrated and moistened peat moss is used as the bed and is rammed down evenly on the seed tray (Fig. 8). The cage is watered adequately from this point. About 100 g of pre-germinated rice seeds is sown on the seed tray (Fig. 9). Enough quantity of rice seeds can increase the reproductive rate for rearing. After sowing, adequate amount of water is supplied and the seed tray is covered with a rectangular transparent acrylic cage (Fig. 9: custom-made cage) and stored in a temperature-controlled room or an incubator maintained at a day length of 16L 8D and a temperature of 25 °C (Fig. 10). To protect the seed tray from infestation by planthoppers, the rearing cages with planthoppers should not be stored in the same room. Water is supplied as needed to keep the seed tray wet.

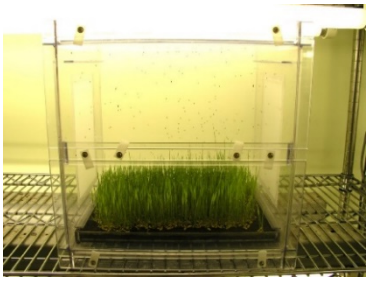


Fig. 7. A transparent acrylic bottle (diameter 70 mm, height 110 mm).

Fig. 8. Peat moss is used as the bed and is rammed down evenly on the seed tray. After sowing, water is supplied adequately.

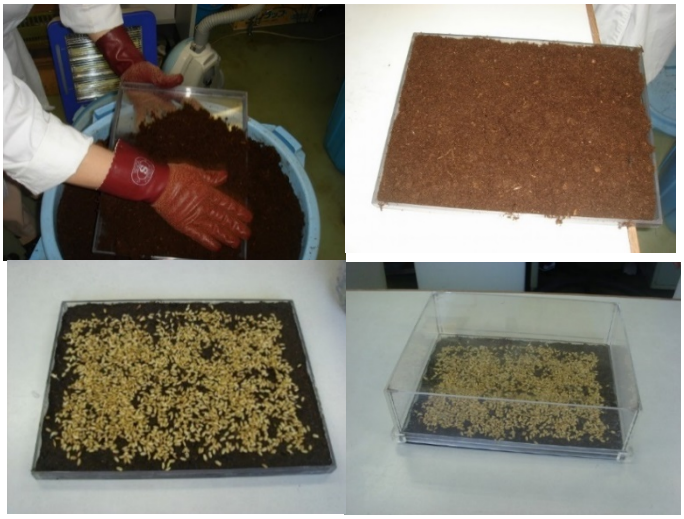


Fig. 9. Pre-germinated rice seeds are sown on the seed tray. The seed tray is covered with a rectangular transparent acrylic cage.

Fig. 10. After sowing, the seed tray is placed under a fluorescent lamp in a temperature-controlled room.

3.3.3 Rearing cycle

Rice seedlings 5 to 10 days after sowing are used for rearing planthoppers. Enough numbers of adult planthopper are introduced into a transparent acrylic cage with rice seedlings and allowed to oviposit for 7 days (Fig. 11). After 7 days, all the adults are removed by using a vacuum cleaner. The rice seedlings with eggs are washed in tap water. After another seven more days, enough number of newly formed nymphs will be hatched. After this point, the old rice seedlings are removed and are replaced with new rice seedlings. Subsequently, rice seedlings are renewed once per week until adult emergence. During the renewal of the rice seedlings, the insect cage is tipped slightly and the insects are beaten down to the bottom of the cage. Old rice seedlings are then removed and new ones are replaced (Fig. 12). The insects that escape from the cage are removed by a vacuum cleaner to prevent contamination of insect strains.

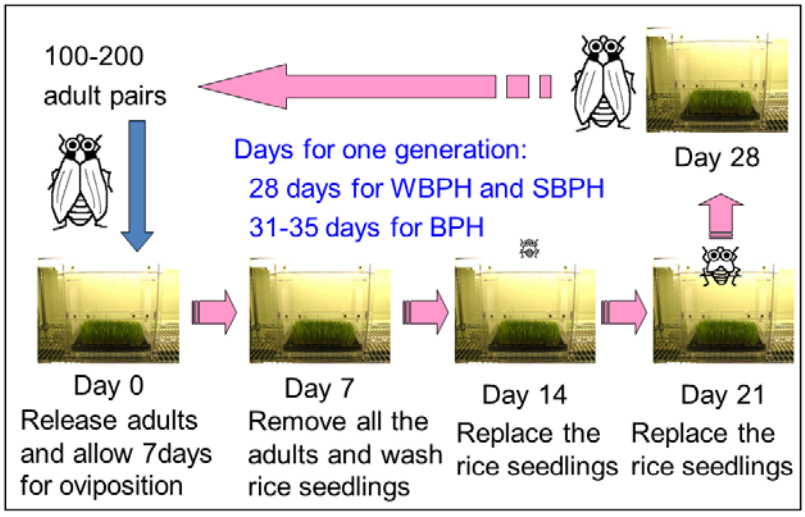


Fig. 11. Rearing cycle of rice planthoppers.

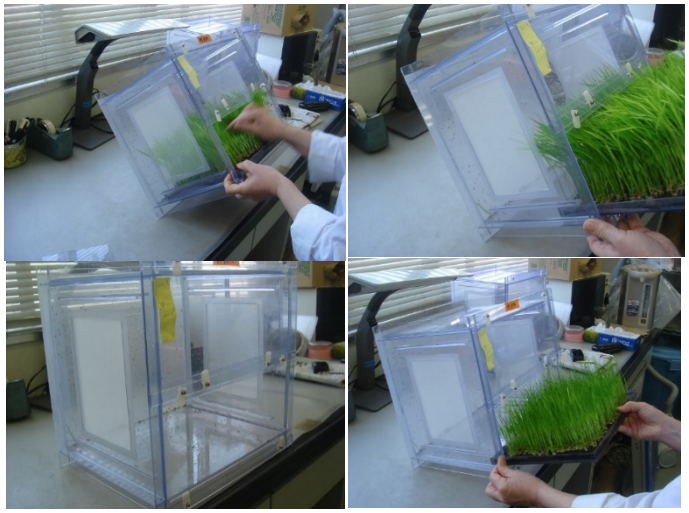


Fig. 12. Replace of rice seedlings.

3.4 Prevention of infestation by insect parasitic fungi

In high humidity rearing condition, the rice planthoppers may be highly parasitized by fungi. To prevent the infection, the rearing cage and rice seedlings should always be kept clean. The rearing cages are washed using chlorine bleach. After 7 days of the start of insect rearing (see section (3)–3), the rice seedlings with eggs are washed in tap water after all the adults are removed using a vacuum cleaner. During washing, the rice seedlings are placed in a large washtub and are washed carefully under running water. If parasitic fungi are abundant, the basal parts of rice seedlings are washed carefully to remove the parasitized dead bodies of planthoppers completely. After washing, the rice seedlings may easily dry up because some amount of peat moss mat is washed out. Therefore, care should be taken to maintain the rice seedlings wet. However, do not overwater the rice seedlings to prevent fungal infection. If the parasitic fungal infection happens at the nymphal stage of mass rearing, use fungicides such as Azoxystrobin and TPN. Other methods such as 1) changing the rice seedlings frequently 2) transferring healthy nymphs to a new rearing cage by a mouth aspirator (the same rearing cage should not be used for a long time), and 3) keeping low humidity in the rearing room, are also effective to reduce the occurrence of fungal infection.

<Attention>

Once insect parasitic fungi have infected the mass-rearing cage, it is difficult to remove them completely. To prevent the occurrence of fungal infection, sterilization of rearing cages and washing of the rice seedlings are recommended as a routine practice even if no infection has been observed previously.

4. Preparations of sample insecticides and concentrations

Label the caps of preservation vials (for example, from 0-10) (Fig. 13). Measure the material weight of the original insecticide by an electronic scale to the nearest 25 mg. Dilute the original insecticide material with acetone to make a 1 % solution of insecticide liquid in vial #0. In case of pymetrozine, the material is diluted with acetone to make a 0.02 % solution of insecticide liquid. Suck up 1 ml acetone using a micro-pipette and pipette into vials #1 to #10. Transfer 1 ml of the 1 % insecticide stock solution into vial #1 and slowly mix it with acetone. Repeat the same procedure to all vials. When insecticides are dissolved in acetone, tips of micro-pipette should be exchanged to prevent contaminations among insecticides. After making the insecticide solutions, cover the caps of vials with parafilm and store in a deep freezer at about –20 ˚C. The standard preservation period for the diluted liquid is generally six months, after which these diluted solutions should be prepared again.

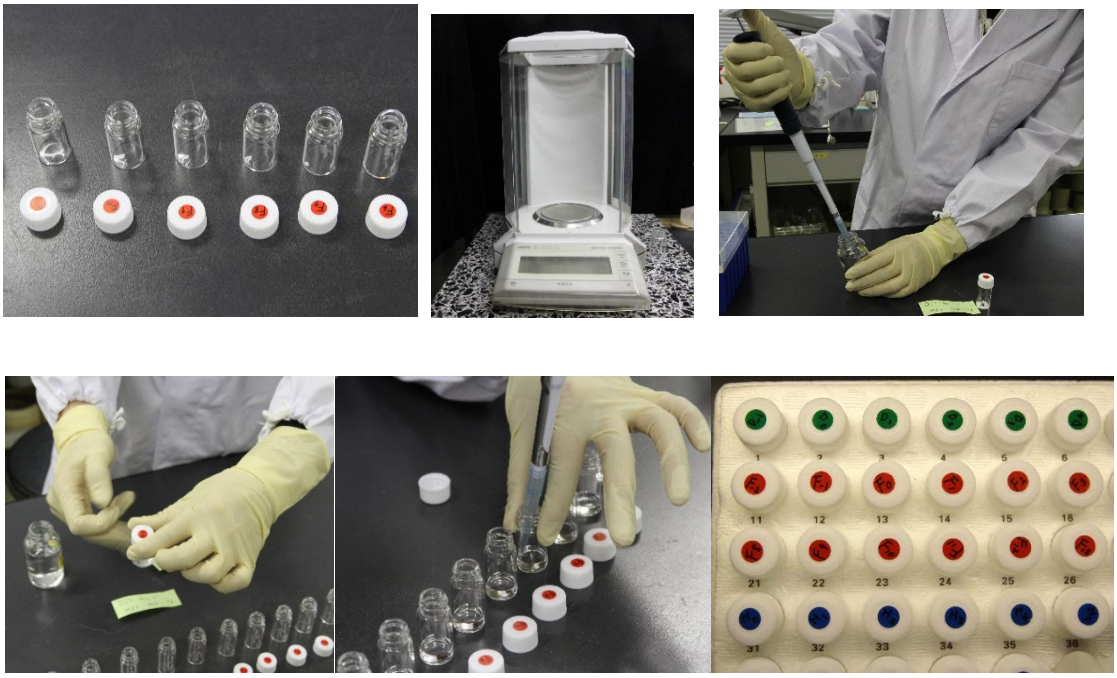


Fig. 13. Preparation of insecticide solutions.

5. Topical application method

5.1 The standard method using a hand micro-applicator

5.1.1. Preparation of acrylic bottle for insect rearing after treatment

Rice seedlings are prepared as food resources for rice planthoppers after topical application because test insects are kept until one to two days after treatment (Fig. 14). Fifteen to twenty rice seedlings grown for seven to ten days after sowing are rolled in tissue papers and are placed in acrylic bottles. If necessary, the rice seedlings are cut to suitable lengths of leaf blades before being placed in the acrylic bottles.

Fig. 14. Preparation of rice seedlings for the bioassay tests.

5.1.2 Preparation of test insects

Adult rice planthoppers within five days after emergence are used for topical application. Fifteen long-winged females are sucked into a glass aspirator and transferred to a glass tube (Fig. 15). Their body weight is calculated from the average value of 15 individuals of test insects. The body weight is used to convert the unit of LD_{50}–value from µg/individuals to µg/g. Test insects are well anaesthetized using carbon dioxide (CO_2) (Fig. 15) in a glass tube before topical application (five to ten seconds by 15L/min). The duration of anesthesia time required is a bit longer for WBPH because they wake up earlier from anesthesia than BPH and SBPH.

Fig. 15. Photo image of a grass tube and a carbon dioxide cylinder.

5.1.3 Treatment of insecticide

Ten µl of insecticide solution is slowly sucked into a 50 µl micro-syringe to prevent formation of air bubbles. After that, the micro-syringe is mounted on a hand micro-applicator (Burkard Manufacturing Co. Ltd., UK) (Fig. 16). The scale per rotation is adjusted to the second from the largest. The liquid droplet size of a hand micro-applicator per rotation is 0.083 µl (calculated from calibration). Before the bioassay, it is confirmed that the insecticide solution can be released smoothly from the tip of the needle. The bioassay is started from the control treatment (only acetone), and then treatment is continued in the sequence from low to high insecticide concentrations. Droplets are applied on the dorsal surface of the thorax of the test insect with 0.083 µl of acetone. At least three replicates of bioassays (15 individuals per replication) are used for each insecticide concentration and control treatment. Each insecticide test consists of five to six insecticide concentrations in the serial dilution and a control. The range of concentrations depends on the level of resistance in tested populations or species. If the approximate LD_{50} value is available for the insecticide to be tested, five or six concentrations around the approximate LD_{50} value as the median concentration are chosen for the bioassay. If no LD_{50} value is available, at least one replication is needed to determine the range of concentrations by trial and error as a preliminary test.

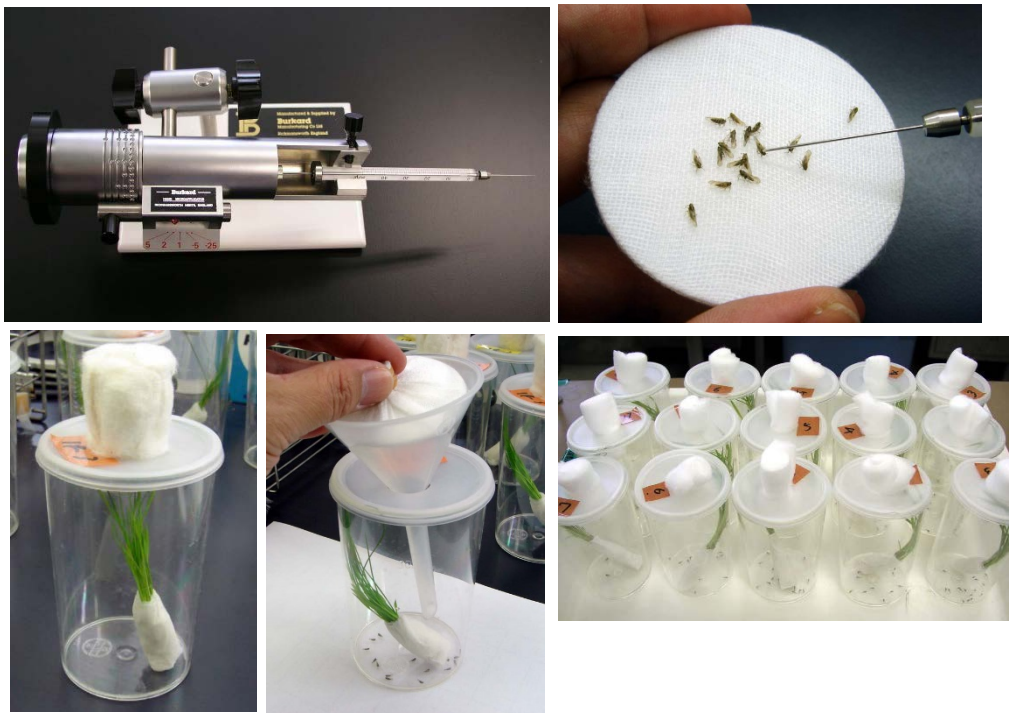


Fig. 16. The standard method: a hand micro-applicator.

5.1.4 Cleaning of the micro-syringe

After the bioassay is completed for an insecticide, the micro-syringe is washed by acetone as soon as possible. First, the insecticide solutions remaining in the micro-syringe are ejected. Next, the tip of the needle is washed by acetone using by a Pasteur pipet. Third, the plunger of the micro-syringe is removed and is washed by acetone. To dilute the residues of insecticide solutions in the barrel, several droplets of acetone are dispensed into the hole of the barrel and ejected from the micro-syringe by pushing the plunger. This procedure is repeated several times. To wash the barrel of the micro-syringe completely, acetone is poured in the small glass vial by a Pasteur pipet, sucked up to fill the inside barrel and ejected from the micro-syringe by pushing the plunger.

5.1.5 Observation after treatment

Bioassay results are recorded 24 h after treatment. The acrylic cage is tapped to drop the insects out on rice seedlings. Rice seedlings are picked up using tweezers. In bioassay tests, the survivor is defined as the individual that shows higher flight activity or the individual in anguish that suffers a leg cramp and grabs the wall of the acrylic bottles. On the other hand, the deceased is defined as the dead insect in the bottom of the acrylic bottles, or the individual in anguish that is not able to stand up or grab the wall of acrylic bottles by oneself. If the mortality is less than 50 % at 24 h after treatment, it is observed again at 48 and 72 h. In that case, rice seedlings should not be picked up and only the numbers of dead individuals are counted at 24 h after treatment.

5.2 The simple method using a repeating dispenser

The simple method is a lower cost method of insecticide application using a Hamilton repeating dispenser compared to the standard method using a hand micro-applicator, because the Burkard hand micro-applicator (Fig. 16) is relatively more expensive (about ten times as expensive) compared to Hamilton repeating dispenser (Fig. 17). The liquid droplet size from a single push of a repeating dispenser equipped with a 10 µl micro-syringe is 0.24 µl, which is three times higher than that of the standard method (0.083µl). The treatment methods and observation of survivor or deceased individuals are similar to the standard method. In SBPH, the mortality tends to be higher in acetone control treatment by the simple method compared to the standard method (Burkard applicator) (Fig. 18). In contrast, there is no difference in mortalities between the standard and simple methods in acetone control treatment in BPH and WBPH. Therefore, the simple method is applied to test insecticide susceptibility only in BPH and WBPH.

Fig. 17. Hamilton repeating dispenser.

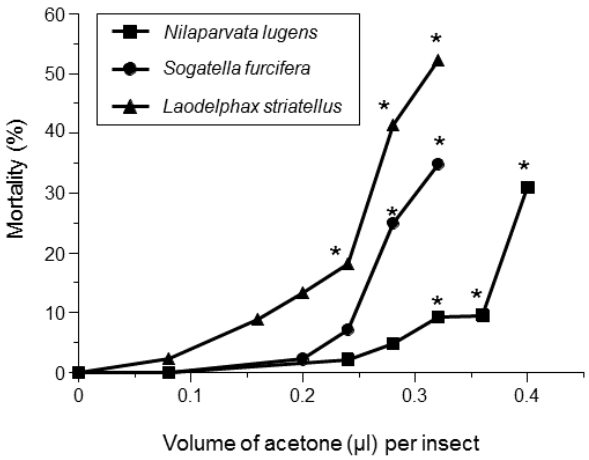


Fig. 18. Mortalities of three rice plant hoppers against acetone in the simple method: repeating dispenser.

5.3 Method for monitoring pymetrozine by combining topical application and number of next generation

Susceptibility monitoring by judging the mortality (LC_{50} or LD_{50}–values), a procedure conventionally adapted for many chemicals (Nagata 1982; Sanada-Morimura and Matsumura 2011), is not available for pymetrozine because it does not show drastically visible lethal action until several days after treatment. Consequently, a susceptibility monitoring method has been developed for BPH using a combination of the rice seedling dipping method and measuring the number of offspring. The Insecticide Resistance Action Committee (IRAC) has adopted this as a standard method (No. 005) (IRAC 2012). The IRAC method can determine the EC_{50} (50 % effective concentration) value, but not the ED_{50} (50 % effective dose) value. Susceptibility monitoring with an ED_{50}–value is a very powerful tool for comparing data collected by researchers in various places and/or times (Matsumura 2014), because the value represents the amount of pesticide directly applied to insects per gram of body weight. Therefore, a new test method to determine ED_{50}–values that is suitable for making comparisons among the accumulating data has been established (Tsujimoto et al. 2016). This method is modified from the IRAC method (No. 005) and is combined with a topical application method.

5.3.1 Rearing tube for the treatment insects

Before the topical application treatment, test-tubes (30 mm outer diameter × height 200 mm) with rice seedling are prepared for rearing treatment insects. Rice seedlings (Japonica var.) are grown in a growth chamber under light with 16L–8D photoperiod at 25 °C for 7–10 days after sowing. The seedlings at the 1–1.5 leaf stage are washed with tap water to remove the soil. Susceptible rice varieties are used for planthoppers. Although Japonica varieties are usually resistant to WBPH, its young rice seedlings are susceptible to them. The roots of 20 plants are wrapped with two sheets of tissue paper or absorbent cotton. The prepared test plants are placed into a test tube (30 mm outer diameter×200 mm height) (Fig. 19). Tap water (or contained liquid fertilizer) (ca. 20 ml) is poured into the test tube until the roots are submerged, and then the excess water and bubbles are removed. About 20 ml of a 1 % (w/v) agar-water solution at 40 °C is poured into the test tube to cover the roots, giving a water-agar depth of about 20 mm (Fig. 20). The test tubes are left for 2–3 h at room temperature for the agar to harden (Fig. 21). Hardened agar prevents the hatched nymph from drowning.

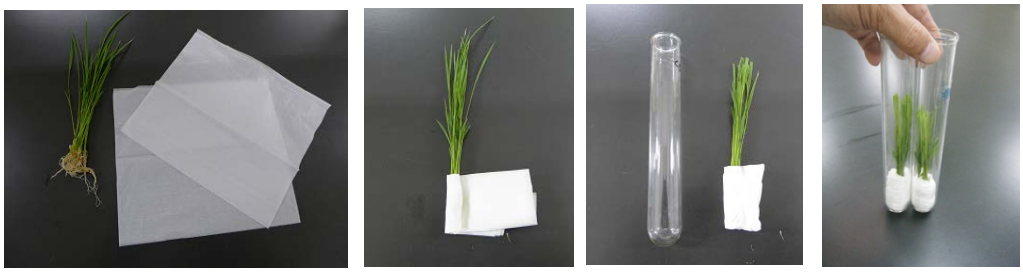


Fig. 19. Preparation of rearing tube for treatment insects.

Fig. 20. Solid agar poured in a test tube.

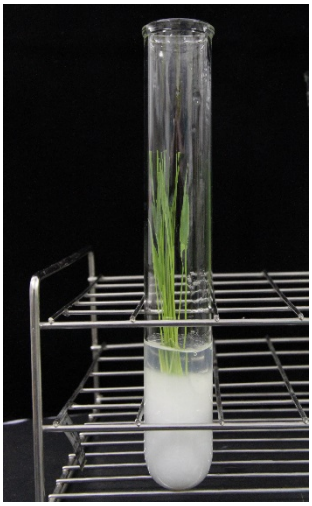


Fig. 21. Test tube with rice seedling.

5.3.2 Topical treatment

Technical-grade pymetrozine (99 %) is dissolved in acetone (0.02 % = 200 ppm) and diluted with acetone to prepare the six test solutions at a concentration of 0.2, 0.4, 0.8, 1.6, 3.1 and 6.3 ppm (see section 4). The treatments contain four replicates for each test solution. A long-winged female adult (within 5 days after emergence) is anesthetized with carbon dioxide for about 5 s, after which the test solution at 0.08 µl/insect is applied topically to the dorsal plate with a hand micro-applicator (Burkard Manufacturing Co., Ltd., UK, or Kiya Seisakusho Ltd., Japan)) (see section 5). Five treated females and three untreated males are placed into test tubes containing rice seedlings that were prepared as described in step (3)–1). The test tube is covered with a silicon lid or a polyester gauze (Toray, Tetron, Japan) lid (Fig. 22). The test is carried out with four or five replicates per treatment. The test tubes are kept under 16L–8D, 25 ℃ and 60 % relative humidity conditions.

Fig. 22. Setting of rearing test tubes under bright growing light.

5.3.3 Counting of the hatched nymphs

All the adult insects are removed 7 days after treatment. After that, the test tubes are rinsed in tap water. The number of offspring is counted 15–16 days after the treatment (8 days after removing the insects) to calculate the 50 % effective dose (ED_{50}) value (Fig. 23). The ED_{50}–value is recalculated based on the body weight of the female adults as shown in the following section.

Fig. 23. Hatched nymphs (left) and ethanol fixed nymphs in test tubes (right).

6. Analysis of results

6.1 Conversion from concentration to dose

For the calculation of LD_{50} or ED_{50}–values, treatment solution volumes (0.08 µl per insect) are converted to doses (weight (insecticide) per weight (insect)) using the following formula (see section 4).

$$Y(\mu g/g) = \frac{a(\mu l) \times b(\%) \times 1{,}000}{100 \times c(g)} = \frac{a(\mu l) \times b(\%) \times 10}{c(g)}$$

Y (µg/g): dose (weight of insecticide (µg) per g of insect)

a (µl): volume of treatment solution per insect (see section 5.1.3))

b (%): concentration of treatment solution

c (g): body weight per insect

6.2 Probit of LD_{50}–values

To calculate the LD_{50}–value, regression slope (b), and the 95 % confidence limit, probit regression analysis is performed between the dose of treatment solution and adjusted mortality using a commercially available software (e.g. PoloPlus (http://leora-software-company-poloplus.software.informer.com/2.0/)).

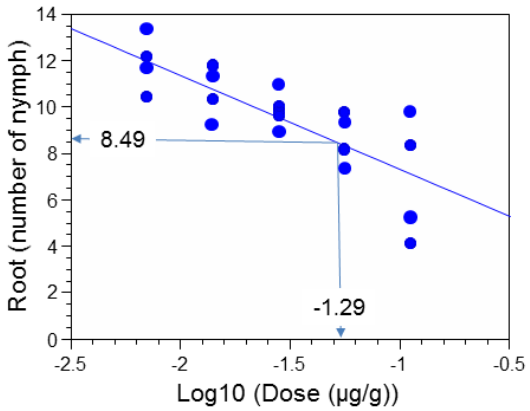
6.3 Calculation of 50 % effective dose (ED_{50}) values for the pymetrozine method

Linear regression analysis was performed between the dose of treatment (logarithmic transformation) and the number of hatched nymphs (square root transformation (number of hatched nymphs + 0.5)) (Fig. 24). ED_{50}–value (i.e., dose at 50 % number of hatched nymphs of control treatment) was calculated by the regression formula (Fig. 25). If the average number of hatched nymphs of control treatment is less than about 100 individuals in BPH and WBPH, or less than about 50 individuals in SBPH (Fig. 24), the experiments must be reworked. This is because the fewer number of hatched nymphs of control treatments should have resulted from some unfavorable condition for the rice seedling during the growing stage. The actual data of ED_{50} values in two BPH strains by this method (Tsujimoto et al., 2016) are shown in Table 1. This method is applicable to the monitoring of pymetrozine not only in BPH but also in WBPH and SBPH (Sanada-Morimura et al., unpublished data).

Conc. (ppm)	Dose (µg/g)	Log_{10} (Dose)	Kumamoto strain							
			No. of nymphs				√(No. of nymphs)			
			Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 1	Rep. 2	Rep. 3	Rep. 4
0	0	-	150	144	122	160	-	-	-	-
0.2	0.007	-2.155	147	130	99	128	12.12	11.40	9.95	11.31
0.4	0.014	-1.854	135	127	111	117	11.62	11.27	10.54	10.82
0.8	0.028	-1.553	121	88	104	75	11.00	9.38	10.20	8.66
1.6	0.056	-1.252	95	77	84	120	9.75	8.77	9.17	10.95
3.1	0.112	-0.951	88	75	70	99	9.38	8.66	8.37	9.95
6.3	0.223	-0.652	59	64	32	67	7.68	8.00	5.66	8.19

Mean number of nymphs in control treatment (0 ppm) = (150+144+122+160)/4 = 144

Fig. 24. Transformation data and mean number of hatched nymphs in control treatment. Numerical values are not actual data in Kumamoto line.



$f(x) = -4.0x + 3.3$ R^2=0.64, P<0.05 (Linear regression analysis)

Square root (50% mean number of nymphs in control treatment)

$= \sqrt{144/2} = 8.49$

$8.49 = -4.0\ x + 3.3$

$x = -1.29$

$Log10(X) = -1.29$

$X = 10^{-1.29} = 0.051$ $\underline{ED_{50} = 0.051\ (ug/g)}$

Fig. 25. Calculation of ED_{50}–value using linear regression analysis.

Table 1 Relationship between number of hatched nymphs and dose of pymetrozine solution and its ED_{50} value of *Nilaparvata lugens* using topical application in vitro rice seedlings

Conc. (ppm)	Osaka 1971 strain					Kumamoto 2011 strain				
	Dose (μg/g)	No. of nymphs[1)]	Suppression rate (%)[2)]	ED_{50} (μg/g) (95%CI)	Slope (*b*)	Dose (μg/g)	No. of nymphs[1)]	Suppression rate (%)[2)]	ED_{50} (μg/g) (95%CI)	Slope (*b*)
0	0	214	-			0	144	-		
0.2	-	-	-			0.007	129	10		
0.4	0.0158	191	10	0.036	-7	0.014	123	14	0.091	-3
0.8	0.0316	90	59	(0.014-0.079)	*P*<0.05	0.0279	100	31	(0.018-1.652)	*P*<0.05
1.6	0.0631	89	59			0.0558	95	34		
3.1	0.1263	56	74			0.1116	82	43		
6.3	-	-	-			0.2232	44	70		

[1)] Mean number of hatched nymphs in a test tube

[2)] Suppression rate (%) = no of nymphs in treated / no of nymphs in untreated X 100

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Appendix: Required equipment

(1) Topical application

•Insecticide (technical products, more than 90 % chemical purity)

•Acetone (>99 % chemical purity)

•Micro-applicator

Ex. 1) Burkard hand micro-applicator (Burkard Manufacturing Co. Ltd., UK) with a micro-syringe (capacity: 50 μl) and an obtuse needle

Ex. 2) Micro-dispenser (PB600–1 Repeating Dispenser, Hamilton Company) with a micro-syringe (capacity: 10 μl) and an obtuse needle

•Screw vial (capacity: 6 ml) (for storage of insecticide solution)

•Micro-pipette (1,000–1,500 μl)

•Plastic funnel (diameter 7 cm, height 130 mm)

•Watch glass (diameter 60 mm) covered by a piece of gauze

•Carbon dioxide (CO_2) in gas cylinder (to anesthetize insects)

•Fine brush, anatomical forceps

(2) Counting of nymphs for Pymetrozine test

•Pymetrozine (technical product, >99 % chemical purity)

•Test tubes (outer diameter 30 mm, height 200 mm)

(7 doses × 4 replicates = 28 tubes with caps (silicon or Tetron polyester gauze (Toray, Tetron) lid))

•Test-tube rack (for use of 50 tubes (diameter 30 mm))

•1 % (w/v) agar-water solution

•Rice seedlings (grown in a greenhouse for 7–10 days after sowing)

•Absorbent cotton (or tissue paper) (for rolling up the roots of rice seedlings)

•50–70 % ethanol (for fixing samples of new nymphs)

•Liquid fertilizer (or water) (for rice seedlings in a test tube)

•Long-handled forceps (length 150 mm)

•Magnifying glass (for counting new nymphs)

•Tally counter (for counting new nymphs)

•Petri dish with square mesh (for counting new nymphs)

•Insect aspirator

(3) Counting of dead insects for normal topical application method

•Acrylic-test bottles (diameter 70 mm, height 110 mm) with rice seedlings (for counting

number of dead insects)

(7–8 doses × 3 replicates = 21 or 24 bottles)

•Cotton caps (diameter 20 mm) (for a breathable cap on a test bottle)

•Long-handled forceps (length 150 mm)

•Magnifying glass (for counting dead insects)

•Insect aspirator

•Tissue paper (for rice seedlings)

(4) Calculation of LD_{50}-value

•Computer program for Probit analysis (e.g. Commercial computer software, PoloPlus Ver. 2) (http://leorasoftware.com/)

(5) Mass rearing of insects and raising rice seedlings

•Acrylic-rearing box (width 150 mm × depth 260 mm × height 340 mm)

•Seedling-raising dish (fitting to the inner diameter of acrylic rearing box)

•Seedling-raising box (width 150 mm × depth 100 mm × height 30 mm) (for rice seedlings in pymetrozine-test tubes)

•Bed soil for seedling-raising dish

•Fungicide for rice seedling (Azoxystrobin, TPN agents etc.)