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Buprofezin susceptibility survey, resistance selection and preliminary determination of the resistance mechanism in *Nilaparvata lugens* (Homoptera: Delphacidae)

Yanhua Wang,[1,2] Congfen Gao,[1] Zhiping Xu,[1] Yu Cheng Zhu,[3]* Jiushuang Zhang,[1] Wenhong Li,[1] Dejiang Dai,[1] Youwei Lin,[1] Weijun Zhou,[1] and Jinliang Shen[1]*

[1] *Key Laboratory of Monitoring and Management of Plant Disease and Insects, Ministry of Agriculture/Department of Pesticide Science, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China*
[2] *Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China,*
[3] *Jamie Whitten Delta States Research Center, ARS-USDA, Stoneville, MS 38776, USA*

Abstract

BACKGROUND: Buprofezin has been used for many years to control *Nilaparvata lugens* (Stål). Assessment of susceptibility change in the insect is essential for maintaining control efficiency and resistance management.

RESULTS: Eleven-year surveys showed that most field populations were susceptible before 2004. However, substantially higher levels of resistance (up to 28-fold) were found in most of the rice fields in China after 2004. A field population was collected and periodically selected for buprofezin resistance in the laboratory. After 65 generations (56 were selected), the colony successfully obtained 3599-fold resistance to buprofezin. Synergism tests showed that *O,O*-diethyl-*O*-phenyl phosphorothioate (SV1), piperonyl butoxide (PBO) and diethyl maleate (DEM) increased buprofezin toxicity in the resistant strain by only 1.5–1.6 fold, suggesting that esterases, P450-monooxygenases and glutathione *S*-transferases had no substantial effect on buprofezin resistance development.

CONCLUSION: The results from this study indicate that *N. lugens* has the potential to develop high resistance to buprofezin. A resistance management program with rotation of buprofezin and other pesticides may efficiently delay or slow down resistance development in the insect. Further investigation is also necessary to understand the resistance mechanisms in *N. lugens*.

Keywords: *Nilaparvata lugens*; buprofezin; susceptibilities; resistance mechanism

1 INTRODUCTION

The brown planthopper, *Nilaparvata lugens* (Stål), is a major rice pest in China and one of the most damaging agricultural insect pests in many parts of Asia.[1,2] Managing the pest with insecticides remains the single most frequently used method, in the absence of other effective methods.[3] Therefore, resistance to various synthetic insecticides has been observed in many locations.[4–6] *Nilapavata lugens* is a migratory insect and is able to travel long distances between the southern and the northern parts of China.[7] This migration may slow down resistance development through dilution, or speed up resistance development in destination areas, depending on the resistance levels in the original areas and the nature of resistance to different insecticides.[8]

Buprofezin, a chitin synthesis inhibitor developed by Nihon-Nohyaku, is a thiadiazine insecticide that is especially effective against Homoptera pests, such as the planthopper, with very low risks to the environment and humans.[9–11] Its mode of action is not fully understood, although the primary effect is to interfere with chitin deposition during molting and to cause nymphal death during ecdysis.[10] In addition, reduced fecundity and egg hatching have been observed after adult females were treated.[12,13] Although buprofezin lacks an acute insecticidal effect, it offers the advantage of longer residual activity against *N. lugens* nymphs than conventional insecticides. Therefore, buprofezin was thought by many researchers to be a unique insecticide for controlling the planthopper, and the registration of buprofezin in the 1980s signaled an important landmark in the chemical control of the pest in China.[14] Buprofezin remained an important chemical for planthopper control until the early 1990s when imidacloprid was introduced for controlling the

* Correspondence to: Yu Cheng Zhu, USDA-ARS-JWDSRC, PO Box 346, Stoneville, MS 38776, USA
E-mail: yc.zhu@ars.usda.gov
Jinliang Shen, Key Laboratory of Monitoring and Management of Plant Disease and Insects, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China
E-mail: jlshen@njau.edu.cn;
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insect.[15] In recent years, buprofezin has again been recommended as one of the main alternatives for replacing methamidophos. Resistance development to buprofezin was first detected in whitefly populations in greenhouses in the Netherlands,[16] and subsequently in northern Europe, Spain and Israel.[17,18] However, because of buprofezin's unique nature, especially its novel mode of action, resistance development in field populations appears to be very slow, and the resistance mechanisms are not well understood.

Because of its long application history and widespread adoption, decreased susceptibility to buprofezin in *N. lugens* has become a major concern in rice-growing regions in China. To provide a foundation for area-wide resistance management of *N. lugens*, the authors initiated a study to investigate regional and temporal changes in susceptibility to buprofezin in rice production areas. In addition, a buprofezin-resistant strain of *N. lugens* was developed by laboratory selection for evaluation of the risk of resistance development and preliminary determination of the resistance mechanisms in *N. lugens*.

2 MATERIALS AND METHODS

2.1 Insects

The susceptible strain of *N. lugens* was originally collected in 1993 from a rice nursery located at the Plant Protection Station of Jiangpu County (Jiangsu, China). The insects were reared on hybrid rice (Shanyou 63), and an isoline was established from a single-pair mating method in the laboratory.

To examine buprofezin resistance in different rice-growing areas, a total of 45 samples were collected from 27 locations in eight different provinces or autonomous regions from 1996 to 2006. Selection of these sites was based on their importance for rice production and their history and intensity of insecticide applications. In addition, these locations were also allocated and considered for further resistance monitoring and analysis. Approximately 800 adults, 500–600 nymphs or sufficient egg masses were collected at each site and transported to the greenhouse on the campus of Nanjing Agricultural University. The insects were reared on insecticide-free hybrid rice (Shanyou 63) before bioassays were performed. The same rice variety at tillering to booting stage was used for maintaining insect colonies and subsequent bioassays. Field-collected insects were mass mated. The third-instar nymphs of F_1 progenies were used for bioassays. All treated insects were maintained at a temperature of 27 ± 1 °C and a 16:8 h light:dark photoperiod.

2.1.1 Anqing population (AQ)

Nilparvata lugens adults of an immigrated generation were collected in 1996 from a rice field of Anqing Academy of Agricultural Science (Anhui, China). The insects were reared for ten generations and then used for resistance selection with buprofezin. G*n* was used to indicate the generations since the first selection with buprofezin.

2.2 Insecticide

A commercial buprofezin 250 g kg^{-1} WP was supplied by Changlong Chemical Industrial Group Co. Ltd (Changzhou, Jiangsu, China) and was used for testing the susceptibility of *N. lugens* during the years from 1996 to 2006. SV1 (*O*,*O*-diethyl-*O*-phenyl phosphorothioate, 500 g L^{-1} EC) was from Changlong Chemical Industrial Group Co. Ltd (Hangzhou, Zhejiang, China). Piperonyl butoxide (PBO, 800 g L^{-1} EC) and diethyl maleate (DEM, reagent grade) were from Acros Organics of America (Morris Plains, NJ).

2.3 Bioassay

The rice-stem dipping method[19–21] was adopted in this study. Rice stems, including roots, were pulled out and washed thoroughly. The basal 10 cm long stems were cut and air dried to remove excess water. Three rice stems were grouped and dipped into appropriate insecticide test solutions for 30 s. Three replicates were used per dose, and 5–6 doses plus water only as the control were used for each chemical. After the rice stems had been air dried for approximately 1 h, moistened cotton was used to wrap the basal end of the rice roots. Treated rice stems were then placed into a 500 mL plastic cup. Twenty third-instar nymphs were introduced into each plastic cup using a suction device. The treated insects were maintained at 27 ± 1 °C and 16:8 h light:dark photoperiod. Mortality was recorded after 120 h. The nymphs were considered dead if they were unable to show coordinated movement after gentle prodding with a fine bristle.

2.4 Resistance selection

Rice stems were treated with buprofezin using the dipping method and were transferred into a cage (57 × 57 × 92 cm). Approximately 1000 third-instar nymphs of *N. lugens* were introduced into the cage and subsequently maintained at 27 ± 1 °C and 16:8 h light:dark photoperiod for 5 days. Survivors were transferred to another cage containing fresh rice seedlings. Pre-trials were conducted to obtain an optimal insecticide concentration for resistance selection. The mortality was controlled to a range between 40 and 70% to ensure sufficient survivors to develop and reproduce enough progenies for the insecticide selection of the subsequent generation. The treatment concentration was approximately the same as the LC_{50} obtained for each generation.

2.5 Test for synergism

To investigate the synergistic effect of major metabolic enzyme inhibitors on the efficacy of buprofezin, SV1, PBO and DEM were individually added to each serial concentration of buprofezin. Pre-trial tests were conducted to determine the highest concentration for each synergist (SV1 5 mg L^{-1}; PBO 20 mg L^{-1}; DEM 500 mg L^{-1}), with no obvious detrimental effects on the third-instar nymphs of either population of *N. lugens*. Other experimental procedures were the same as described above. To assess the degree of synergism, the synergistic ratio (SR) was calculated by dividing the LC_{50} value of buprofezin alone by the LC_{50} value of buprofezin plus synergist.

2.6 Data analysis

The POLO program was used for probit analysis of dose–response data,[22] unless otherwise stated. Mortality was corrected using Abbott's formula for each probit analysis.[23] The resistance ratio (RR) was calculated by dividing the LC_{50} of a field population by the LC_{50} of the susceptible strain. Significant level of mean separation ($P < 0.05$) was based on non-overlap between the 95% confidence limits (CL) of two LC_{50} values. Resistance levels were classified on the basis of the standard described by Shen and Wu[24] as susceptible (RR < 3-fold), minor resistance (RR = 3–5-fold), low resistance level (RR = 5–10-fold), medium resistance level (RR = 10–40-fold), high resistance level (RR = 40–160-fold) and extremely high resistance level (RR > 160-fold).

3 RESULTS

3.1 Dose response and resistance level in field populations

The LC_{50} value of buprofezin in the susceptible strain was 0.268 (0.21–0.32) mg AI L^{-1} (Tables 1 and 2). A total of 45 field samples collected from eight provinces were examined from 1996 to 2006 for their susceptibility to buprofezin. The results showed that most of the *N. lugens* populations from Jiangsu (Nanjing, Yizheng and Nantong), Anhui (Anqing) and Guangxi (Guilin) remained susceptible (RR < 3) to buprofezin from 1996 to 2004, except for the Anqing and Nanjing populations with minor resistance (RR = 3.3- and 3.5-fold respectively) in 1996. However, a medium resistance level to buprofezin (RR = 28.1) was found in the Nanjing population in 2005 (Table 2), where the insects were repeatedly assayed from 1996 to 2004 and their resistance ratios were no more than 5-fold. The other 14 populations from Jiangsu, Zhejiang, Anhui and Jiangxi provinces also developed low resistance levels to the insecticide (RR = 5.9–9.7) in 2005. Further resistance surveys in 2006 (Table 2) indicated that twelve field populations of *N. lugens* from Guangxi, Jiangxi, Jiangsu, Hubei, Zhejiang, Anhui and Fujian provinces developed minor to low-level resistance to buprofezin (RR = 2.5–9.4), which did not increase substantially but maintained median levels similar to those during the same period in 2005.

3.2 Buprofezin resistance selection

For the selection tests, the experiments lasted 65 generations. Fifty-six generations were treated with buprofezin, and the other nine generations (F6-F9, F15-F17 and F19-F20) were not treated with the insecticide. Results of the buprofezin resistance selection (Fig. 1) showed that resistance ratios varied substantially over the 65-generation selection period. In the first 25 generations, selection with buprofezin did not reveal a distinct increase in resistance ratios, which only fluctuated between 1.5 and 3.9. From generation 26 to generation 32, the resistance ratios quickly increased to 1037.3-fold. After that, the resistance ratios continued to increase at a relatively slow pace until generation 53 (G53), which obtained a 1658.2-fold resistance ratio. A rapid increase in resistance ratios appeared again in the subsequent 12 generations. Generation 65 (G65) showed the highest resistance to buprofezin (RR = 3598.9-fold) in the total 65 generations tested. It was likely that the resistance level of *N. lugens* to buprofezin would have increased further if the selection had continued.

Table 1. Dose response and resistance ratio (RR) to buprofezin in field populations of *Nilaparvata lugens* collected from 1996 to 2004

Test populations			Dose response		
Years	Provinces	Locations	Slope (±SE)	LC_{50} (mg AI L^{-1}) (95%CI)	RR
Susceptible strain			2.89 (±0.23)	0.268 (0.21–0.32)	1.0
1996	Anhui	Anqing	4.05 (±0.48)	0.90 (0.81–0.96)	3.3
	Guangxi	Guilin	2.77 (0.19)	0.47 (0.36–0.56)	1.7
	Guangxi	Nanning	4.95 (±0.37)	0.77 (0.72–0.83)	2.9
	Jiangsu	Nanjing	1.66 (±0.14)	0.95 (0.78–1.30)	3.5
1997	Anhui	Anqing	3.30 (±0.28)	0.67 (0.61–0.73)	2.5
	Guangxi	Guilin	2.44 (±0.19)	0.53 (0.44–0.61)	2.0
	Guangxi	Nanning	2.71 (±0.23)	0.81 (0.77–0.86)	3.0
	Jiangsu	Nanjing	4.78 (±0.51)	0.59 (0.41–0.62)	2.2
1998	Guangxi	Nanning	2.71 (±0.22)	0.38 (0.32–0.44)	1.4
	Jiangsu	Yizheng	4.73 (±0.57)	0.43 (0.38–0.47)	1.6
1999	Guangxi	Nanning	2.38 (±0.23)	0.44 (0.39–0.49)	1.6
	Jiangsu	Nantong	2.04 (±0.17)	0.38 (0.32–0.42)	1.4
2001	Jiangsu	Nanjing	1.92 (±0.14)	0.39 (0.32–0.47)	1.5
2002	Guangxi	Nanning	2.29 (±0.25)	0.67 (0.58–0.76)	2.5
2003	Jiangsu	Nanjing	2.09 (±0.18)	0.48 (0.40–0.58)	1.8
2004	Jiangsu	Nanjing	2.60 (±0.23)	0.61 (0.52–0.72)	2.3

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Table 2. Dose response and resistance ratio (RR) to buprofezin in field populations of *Nilaparvata lugens* collected in 2005 and 2006

Test populations				Dose response		
Year	Month	Provinces	Locations	Slope (± SE)	LC_{50} (mg AI L^{-1}) (95% CI)	RR
2005	August	Guangxi	Guilin	2.15 (±0.21)	2.18 (1.76–2.60)	8.1
2005	August	Guangxi	Nanning	2.07 (±0.20)	1.59 (0.84–2.60)	5.9
2005	August	Hunan	Changde	1.87 (±0.24)	2.60 (1.51–3.60)	9.7
2005	August	Jiangsu	Nanjing	1.64 (±0.18)	7.54 (5.86–9.63)	28.1
2005	September	Anhui	Caohu	1.47 (±0.12)	1.76 (1.42–2.01)	6.6
2005	September	Jiangsu	Gaochun	1.56 (±0.16)	2.01 (1.59–2.60)	7.5
2005	September	Jiangsu	Suzhou	1.46 (±0.13)	1.68 (1.34–2.01)	6.3
2005	September	Jiangsu	Wuxi	1.62 (±0.17)	2.09 (1.51–2.93)	7.8
2005	September	Zhejiang	Haiyan	1.46 (±0.16)	1.59 (1.26–2.01)	5.9
2005	September	Zhejiang	Jiaxing	1.62 (±0.18)	2.09 (1.68–2.68)	7.8
2005	September	Zhejiang	Shaoxing	1.99 (±0.19)	2.01 (1.68–2.43)	7.5
2005	September	Zhejiang	Tongxiang	1.31 (±0.16)	2.43 (1.84–3.18)	9.1
2005	September	Zhejiang	Yuyao	1.84 (±0.22)	1.68 (1.34–2.18)	6.3
2005	October	Anhui	Hexian	1.34 (±0.16)	1.93 (1.42–2.51)	7.2
2005	October	Jiangxi	Nanchang	1.30 (±0.20)	1.68 (1.26–2.26)	6.3
2005	October	Jiangxi	Xinjian	1.74 (±0.19)	1.59 (1.26–2.01)	5.9
2006	June	Guangxi	Nanning	2.75 (±0.25)	2.51 (2.18–2.93)	9.4
2006	July	Jiangxi	Shanggao	2.57 (±0.28)	1.09 (0.92–1.34)	4.1
2006	August	Hubei	Xiaogan	2.57 (±0.28)	1.09 (0.84–1.34)	4.1
2006	August	Jiangsu	Nanjing	2.38 (±0.25)	1.51 (1.26–1.84)	5.6
2006	August	Zhejiang	Jinhua	2.07 (±0.21)	0.67 (0.50–0.84)	2.5
2006	September	Anhui	Hexian	2.04 (±0.21)	1.51 (1.27–1.84)	5.6
2006	September	Anhui	Ningguo	2.09 (±0.21)	1.51 (1.27–1.68)	5.6
2006	September	Anhui	Qianshan	2.33 (±0.26)	2.26 (1.84–2.68)	8.4
2006	September	Fujian	Fuqing	2.07 (±0.20)	1.17 (0.84–1.59)	4.4
2006	September	Jiangsu	Tongzhou	2.73 (±0.28)	1.59 (1.34–1.93)	5.9
2006	September	Zhejiang	Haiyan	2.30 (±0.25)	1.59 (1.34–1.93)	5.9
2006	October	Jiangsu	Dongtai	2.04 (±0.20)	1.68 (1.42–1.93)	6.3

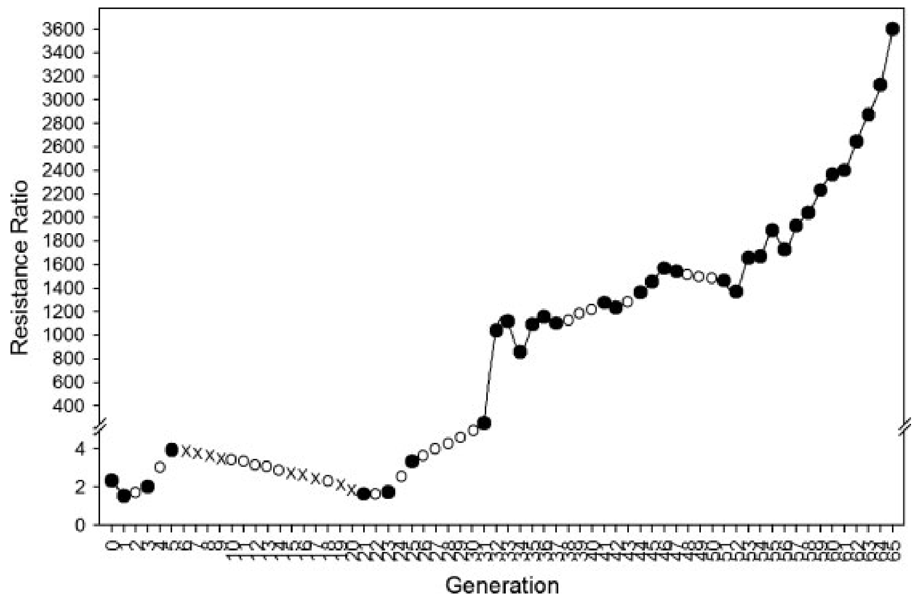

Figure 1. Change in resistance ratios to buprofezin in *N. lugens* under different selection pressures. Among the 44 generations treated with buprofezin, 22 generations were measured for LC_{50} and RR (●), and 22 generations were not measured for RR (○). Nine generations were not treated with buprofezin (×). The o and × points are for connection only and have no RR value.

3.3 Effect of synergists on buprofezin resistance

SV1, PBO and DEM inhibit esterases and P450 monooxygenases, P450 monooxygenases and glutathione *S*-transferases respectively. The inhibition of these metabolic enzymes can enhance insecticide toxicity. Synergistic effects of SV1, PBO and DEM on buprofezin were tested on the susceptible and G65 resistant strains of *N. lugens*. The results (Table 3) indicate that synergistic ratios of SV1, PBO and DEM

Table 3. Synergistic effects of SV1, PBO and DEM on buprofezin in susceptible (S) and buprofezin-selected G65 strains of *Nilaparvata lugens*

Strain	Treatment	Slope	LC_{50} (mg AI L^{-1}) (95% CI)	Synergism ratio	Relative synergism ratio[a]
S	Buprofezin	2.3475	0.268 (0.21–0.32)	1	–
S	Buprofezin + SV1	2.3182	0.14 (0.11–0.17)	1.9	–
S	Buprofezin + PBO	2.8187	0.17 (0.13–0.20)	1.6	–
S	Buprofezin + DEM	2.7732	0.19 (0.15–0.23)	1.4	–
G65	Buprofezin	1.9310	964.50 (755.77–1151.65)	1	–
G65	Buprofezin + SV1	1.5811	321.50 (251.92–383.88)	3.0	1.6
G65	Buprofezin + PBO	1.8748	401.88 (314.90–479.85)	2.4	1.5
G65	Buprofezin + DEM	1.9025	459.29 (359.89–548.40)	2.1	1.5

[a] Relative synergism ratio = synergism ratio of resistant strain/synergism ratio of susceptible strain.

were 1.9, 1.6 and 1.4-fold in the S strain, and 3.0, 2.4 and 2.1-fold in the G65 resistant strain respectively. The relative synergism ratios (synergism ratio in the G65 resistant strain divided by the synergism ratio in the susceptible strain) were 1.6-, 1.5- and 1.5-fold for SV1, PBO and DEM respectively. These results indicate that esterases, P450-monooyxgenases and glutathione *S*-transferases had no substantial effect on buprofezin resistance development in *N. lugens*.

4 DISCUSSION

Before the mid-1990s, buprofezin, along with carbamates and organophosphates, played an important role in *N. lugens* control.[2,4,25] Owing to its unique action on the ecdysis of the nymphal stage,[26] buprofezin became more valuable in chemical control of the planthoppers. However, imidacloprid, first introduced in the early 1990s in China, quickly became a dominant insecticide owing to its systemic and relatively fast action against the insect,[27] which resulted in an apparent decrease in buprofezin applications.[28] However, the development of a high to extremely high level of resistance to imidacloprid in *N. lugens* was found in many rice fields in 2005 (data not shown). Therefore, buprofezin was recommended again as the main insecticide for planthopper control.

Nilaparvata lugens remained susceptible to minor resistance levels to buprofezin from 1996 to 2004, but low to medium resistance levels were found in the majority of rice fields in 2005. Widespread and long-term usage of buprofezin was probably the main reason for the development of a medium resistance level to the insecticide in *N. lugens*. Similar situations of resistance increase due to extensive and intensive applications have also been observed in many other insects, such as imidacloprid resistance development in *Leptinotarsa decemlineata* (Say) and *Bemisia tabaci* (Gennadius), triazophos resistance in *Chilo suppressalis* (Walker) and pyrethroid resistance in *Helicoverpa armigera* (Hübner).[22,29–32]

Many factors might be associated with the resistance increase in *N. lugens*. In terms of outbreak frequency, the planthopper is classified as an intermediate outbreaking pest with 2–3 population outbreaks every 10 years in China.[33] Buprofezin was used once a year when the population density was low, and 2–3 times each year when the population density increased to a serious level. In spite of that, buprofezin was still a less prevalently used insecticide than imidacloprid and other insecticides for controlling *N. lugens* after the 1990s.[28] Secondly, *N. lugens* is a typical long-distance migratory pest, and the inheritance of resistance to buprofezin was incompletely recessive.[34] After migration, heterozygous (susceptible) offspring are produced at destination sites, considering the emigrating and resident populations to have different genotypes (susceptibilities) to the insecticide. Therefore, migration plays a substantial role in delaying resistance development through diluting high-level resistance.[35] So, infrequent use of buprofezin and migration might have effectively kept the resistance development under control until 2004, when only a minor level of resistance was detected in many rice-growing areas. The resistance increase since 2005 may be associated with intensified application of buprofezin after the insect became highly resistant to imidacloprid, the dominant insecticide for planthopper control.

Owing to the incompletely recessive nature of the resistance to buprofezin, the authors' eleven-year surveys indicated that the rate of resistance development to buprofezin was much slower than resistance development to imidacloprid in *N. lugens*. Current resistance development to buprofezin might be at an early stage or in a slow increase phase. Whether or not a rapid increase will happen sooner or later depends on selection pressure, e.g. application intensity in the field.

Rotating buprofezin with other insecticides (such as chlorpyrifos, fipronil, isoprocarb, dichlorovos, pymetrozine and many others) with different modes of action can reduce selection pressure and delay or slow down resistance development in *N. lugens*. The present laboratory selection data (Fig. 1) showed a slow increase phase and a fast increase phase. Because the first 20 generations received low selection pressure (non-continuous selection), the resistance development was relatively slow. Resistance ratios also tended to fluctuate with the short term of termination and resumption of selection. Unlike the early generations, generations 21 to 53 received continuous selection of buprofezin. Subsequently, the resistance levels increased at a substantially faster pace than those of the early generations.

The results also showed that the planthopper has the potential to achieve an extremely high level of resistance if a population receives high selection pressure, as seen in the laboratory during the continuous selection experiment. The present resistance selection data also suggest that, if this laboratory phenomenon could represent a field situation, alternation and rotation of buprofezin with other classes of insecticides would certainly delay and slow down resistance development to buprofezin in *N. lugens*. An effective area-wide resistance management program would be achievable if buprofezin were properly rotated with carbamates, organophosphates and other novel insecticides not only in immigration regions but also in emigration regions.

A previous study suggested that buprofezin resistance development in *N. lugens* might be associated with target-site alterations.[36] Considering that a specific synergist (enzyme inhibitor) is a useful tool for understanding the possible underlying mechanisms of insecticide resistance, SV1, PBO and DEM were first used in this study to determine whether the resistance in *N. lugens* was associated with major metabolic detoxification enzymes. The results suggested that esterases, P450-monooxygenases and glutathione *S*-transferases had no substantial effect on buprofezin detoxification and resistance development in *N. lugens*. Because the synergists in this study did not eliminate resistance substantially in the resistant strain, other mechanisms might be involved in resistance development in *N. lugens*. With a resistant strain established in the authors' laboratory, further studies will be carried out to reveal details of biochemical and molecular aspects of the resistance.

In summary, buprofezin was recently recommended as one of the alternatives for replacing highly toxic organophosphorus insecticides for controlling economically important insects on rice. The results from this study caution that the planthopper is able to achieve an extremely high level of resistance to nullify the effectiveness of buprofezin. Eleven-year resistance monitoring data showed that the resistance ratio in *N. lugens* tended to increase over that period. The relatively slow pace of resistance development may be attributed to some influential factors such as recessiveness, fitness costs of resistance and the migratory nature of the insect. Reducing selection pressure on the target insect through rotation with other functionally different insecticides might be a key component for delaying and minimizing resistance risk. In addition, biochemical and molecular characterization of the resistance mechanisms is necessary in future studies to develop techniques and discover effective inhibitors against the target enzymes that are responsible for resistance. By understanding source populations and dynamics of migration, it may be possible to predict the impact on resistance development in destination areas.

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