

Cry1Ab Rice Does Not Impact Biological Characters and Functional Response of *Cyrtorhinus lividipennis* Preying on *Nilaparvata lugens* Eggs¹

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ABSTRACT

One concern about the use of transgenic plants is their potential risk to natural enemies. In this study, using the eggs of the rice brown planthopper, *Nilaparvata lugens*, as a food source, we investigated the effects of Cry1Ab rice on the biological characteristics and functional response of an important predator *Cyrtorhinus lividipennis*. The results showed that the survival ability (adult emergence rate and egg hatching rate), development (egg duration, nymphal developmental duration), adult fresh weight, adult longevity and fecundity of *C. lividipennis* on Bt rice plants were not significantly different compared to those on non-Bt rice plants. Furthermore, two important parameters of functional response (instantaneous search rate and handling time) were not significantly affected by Bt rice. In conclusion, the tested Cry1Ab rice does not adversely impact the biological character and functional response of *C. lividipennis*.

Keywords: Bt rice; safety assessment; non-target organism; natural enemy

Cry1Ab 水稻不影响黑肩绿盲蝽的生物学特征以及对褐飞虱卵的捕食功能反应

中文摘要:

使用转基因作物的担忧之一就是它们对天敌的潜在风险。本文以褐飞虱的卵为食物,考察了 Cry1Ab 水稻对稻田重要天敌黑肩绿盲蝽的生物学特征和捕食功能反映的影响。结果显示在 Bt 水稻上饲养取食褐飞虱卵的黑肩绿盲蝽的存活率、卵孵化率、卵发育历期、若虫发育历期、成虫体重、寿命和繁殖力与在非 Bt 水稻上饲养的黑肩绿盲蝽没有显著差异。同时,黑肩绿盲蝽对褐飞虱卵的捕食功能反应的 2 个重要参数(瞬时搜寻率和处理时间)不受 Bt 水稻的影响。综上所述,供试的 Cry1Ab 水稻不会对黑肩绿盲蝽的生物学特征和捕食功能反应造成不利的影响。

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关键词: Bt 水稻; 安全性评价; 非靶标生物; 天敌

1. INTRODUCTION

Many varieties of Bt rice have been developed for managing rice stem borers and leaffolders in the laboratory (Cheng *et al.* 1998; Husnain *et al.* 2002; Li *et al.* 2004; Ho *et al.* 2006; Kim *et al.* 2008; Zaidi *et al.* 2009), and some of them showed high efficacy against lepidopteran pests in the field (Ye *et al.* 2001, 2003; Bashir *et al.* 2004; Breitler *et al.* 2004). One Bt rice cultivar (Huahui 1) and its hybrid line (Bt Shanyou 63) have been approved for limited commercialization testing in Hubei Province in China for a 5-year period (2009-2014) (MAPRC 2009). With the introduction of genetically modified insect-resistant crops as a new pest management approach, the effects of Bt rice on non-target organisms and the environment continue to be debated.

One concern regarding potential risks of Bt rice is its effect on non-target herbivores and natural enemies. The effects of Bt rice on the biological characteristics and population dynamics of several important non-target herbivorous pests, including the rice planthopper (Bernal *et al.* 2002; Fu *et al.* 2003; Chen *et al.* 2007; Chen *et al.* 2009; Chen *et al.* 2012), rice leafhopper (Chen *et al.* 2006), and rice thrips (Akhtar *et al.* 2010) have been evaluated under laboratory and field conditions. Studies about the impacts on natural enemies have been focused on their population dynamics (Chen *et al.* 2003; Chen *et al.* 2007; Han *et al.* 2011), abundance, and diversity (Li *et al.* 2007; Bai *et al.* 2012) in the fields. Moreover, there were studies examining the development and reproduction of natural enemies on Bt rice (Bai *et al.* 2006; Tian *et al.* 2012).

Cyrtorhinus lividipennis Reuter (Hemiptera: Miridae) occurs in rice fields in Southeast Asia, and preys mainly on eggs or young nymphs of the brown planthopper, *Nilaparvata lugens* (Stål), white-backed planthopper, *Sogatella furcifera* (Horváth) and the rice green leafhopper, *Nephotettix virescens* Distant (Lou *et al.* 1996), by piercing and sucking out their juices (Reyes and Gabriel 1974). *C. lividipennis* population dynamics shows synchronization with the occurrence of *N. lugens* (Wu and Chen, 1987; Wang *et al.* 2011) and is considered as a good biological control agent in rice paddies especially for *N. lugens*, which is one of the most destructive pests of rice in Asia. Insecticidal proteins in Bt rice may negatively impact natural enemies indirectly exposed through the food web (Jiang *et al.* 2004). A previous study showed that there were no differences in nymphal survival and development duration (to adult) between *C. lividipennis* fed on *N. lugens* nymphs reared on Bt rice and that reared on non-Bt rice (Bernal *et al.* 2002). However, no studies were carried out using *N. lugens* eggs, which contain

Bt protein when laid in Bt rice (Gao 2010) and is the main food source for *C. lividipennis*, to evaluate the effect of Bt rice on *C. lividipennis*.

In the present study, we used primarily *N. lugens* eggs as the food resource for *C. lividipennis* and evaluated: (1) Bt protein uptake by *C. lividipennis*; (2) Cry1Ab rice effects on the development and reproduction of *C. lividipennis*; and (3) Cry1Ab rice effects on the functional responses of *C. lividipennis*.

2. RESULTS

2.1 Uptake of Bt proteins by *C. lividipennis*

The average concentration of Cry1Ab in *C. lividipennis* adults after feeding on Bt rice for 5 d was 50.7 ng g⁻¹ fresh weight (FW) (equaling to 1.85% of the total expressed in Bt rice stem), while the average concentration of Cry1Ab in the Bt rice stems was 2.74 µg g⁻¹ FW. Only trace level of Cry1Ab protein (less than the quantification level of 0.3125 ng g⁻¹, but significantly higher than the negative control) was detected in *N. lugens* eggs produced on the Bt rice plants. As expected, no Bt protein was detected in samples taken from the non-Bt rice plants.

2.2 Effects of Bt rice on *C. lividipennis* survival, development and reproduction

No adverse effect of Bt rice was observed for various parameters of *C. lividipennis* survival, development and reproduction. The average emergence rate of *C. lividipennis* fed on *N. lugens* eggs laid on KMD1 (Bt rice) and Xiushui 11 (non-Bt rice) was 75.33% and 61.33%, respectively, and did not differ significantly. The average hatch rate of *C. lividipennis* eggs laid on Bt rice and non-Bt rice was 89.46% and 86.86%, respectively, and did not differ significantly. The average egg duration for *C. lividipennis* on Bt rice and non-Bt rice was about 9 d with no significant difference. The nymphal development duration of female or male of *C. lividipennis* also did not show any significant difference between those fed on *N. lugens* eggs laid on Bt rice and on non-Bt rice. The fresh weight of female or male *C. lividipennis* fed on *N. lugens* eggs laid on Bt rice was not significantly different from those fed on *N. lugens* eggs laid on non-Bt rice. The longevity of male or female *C. lividipennis* adults showed no significant difference between those fed on *N. lugens* eggs laid on the Bt and on non-Bt rice plants. The average number of eggs laid by female *C. lividipennis* that fed on *N. lugens* eggs laid on Bt rice or on non-Bt rice was 177.6 and 181.8, respectively, with no significant difference (Table 1).

2.3 The effect of Bt rice on the functional responses of *C. lividipennis*

The functional response of *C. lividipennis* evaluated with two situations, where the *C. lividipennis* was

reared on TN1 with *N. lugens* until being used in a response test to *N. lugens* eggs laid on Bt rice or non-Bt rice (Test 1), or the *C. lividipennis* were reared on Bt or non-Bt rice with *N. lugens* eggs until being used in a response test with *N. lugens* eggs laid on TN1. In both tests, the functional response data were fitted to Holling Type II with $R^2 > 93\%$ (Table 2, Fig. 1). The two important functional response parameters, instantaneous search rate (a) and handling time (b) were not significantly affected by Bt rice (Test 1: $t=1.03-1.72$, $df=4$, $P=0.36-0.16$; Test 2: $t=0.88-1.42$, $df=4$, $P=0.43-0.23$) (Table 2).

3. DISCUSSION

With the Chinese government's approval for commercializing testing of two Bt rice lines in Hubei province from 2009-2014 (MAPRC 2009), Bt rice is on the verge of being commercially released in China. Data on potential effects of Bt rice on non-target arthropods are needed prior to commercialization. *N. lugens* is one of the most destructive rice pests that are not targeted by Bt proteins. With ca 50% of rice paddy fields in China are infested with *N. lugens* every year since the 1980s (Cheng *et al.* 2003), it is essential to study the performance of natural enemies of *N. lugens* on Bt rice before commercialization.

The green mirid bug, *C. lividipennis*, is an important natural enemy of *N. lugens* and plays a significant role in suppressing *N. lugens* populations, especially with expected reduction of chemical sprays by Bt rice. Bernal *et al.* (2002) reported that *C. lividipennis* was not impacted by Cry1Ab rice when using *N. lugens* nymphs as preys. However, *C. lividipennis* prefer *N. lugens* eggs over nymphs and develop better when they prey on eggs (Chua and Mikil 1989). Studies have showed that *N. lugens* eggs contain a detectable amount of Bt protein when they were laid in Bt rice (Gao 2010; An 2013). Thus, in the present study, we evaluated the potential effects of Bt rice on *C. lividipennis* using *N. lugens* eggs as a food source.

In our study, only a trace amount of Bt protein was detected in *N. lugens* eggs laid in Bt rice. However, the tested *C. lividipennis* contained a level of Cry1Ab protein that was much higher than the detection limit. *C. lividipennis* is a zoophytophagous predator, i.e., it also sucks up rice plant sap when it preys on *N. lugens* eggs. Thus, the Bt protein in *C. lividipennis* also might be from the Bt rice plants. Another way that *C. lividipennis* is exposed to Bt protein is from *N. lugens* honeydew as an additional food source (Matsumura and Suzuki 1999) that contains a detectable level of Bt protein (Bernal *et al.* 2002). Although *C. lividipennis* may be impacted by exposure to Bt protein, the potential adverse effect of the Cry1Ab protein in *C. lividipennis* (a 54-fold lower than that in Bt rice) was minimum as indicated by the current study. This result agreed with most reported studies, where no

bioaccumulation and biomagnification of Bt protein or adverse effect were found for nontarget arthropods (Li *et al.* 2011; Meissle and Romeis 2012; Tian *et al.* 2012, 2014).

Although Bt protein was transferred to *C. lividipennis* through tritrophic interactions, no significant difference in the biology and reproduction of *C. lividipennis* was found between the Bt rice treatment and non-Bt rice treatment when using *N. lugens* eggs as preys (Table 1). Similar results were reported by Bernal *et al.* (2002) that no difference was detected between Bt rice and non-Bt rice treatments using *N. lugens* nymphs as preys. However, the development time of *C. lividipennis* feeding with *N. lugens* eggs (approximate 9.5 d in the present study) was dramatically shorter than that of those fed on *N. lugens* nymphs (ca 17 d, Bernal *et al.* 2002). It indirectly confirmed that *C. lividipennis* grows better on *N. lugens* eggs as a preferred prey. Our results were consistent with previous studies on natural enemies exposed to Bt proteins expressed by Bt rice. A spider, *Pardosa pseudoannulata* (Bösenberg *et Strand*), (Tian *et al.* 2012), a ladybird beetle, *Propylea japonica* (Thunberg), (Bai *et al.* 2006) and a green lacewing, *Chrysoperla sinica* (Tjeder), (Bai *et al.* 2005) were not affected by Bt rice. Our results are also consistent with field investigations that demonstrated the population dynamics of *C. lividipennis* in Bt rice fields were not significantly different from those in the non-Bt rice fields (Han *et al.* 2011; Xu *et al.* 2011).

Assessments of Bt crop effect on natural enemies often focus on investigating the biological characters of the animals of the interest with exposure to Bt protein (Romeis *et al.* 2008; Lundgren *et al.* 2009; Naranjo 2009). However, impacts on the biology of a given natural enemy do not necessarily reflect the impacts on its ecological functionality, e.g. predation, which is more important. For example, rice under a high nitrogen regime could improve the biological performance of *C. lividipennis* (including shorter development time, heavier weight and higher fecundity) (Lu 2003); however, the predation capabilities of *C. lividipennis* on *N. lugens* eggs and nymphs were negatively correlated with the nitrogen content of rice plants (Lu *et al.* 2005). Thus, the potential effects of Bt rice on *C. lividipennis* should be evaluated directly focusing on ecological functionalities, even when no significant differences in the biology of *C. lividipennis* were detected between Bt rice and non-Bt rice treatment. In the present study, the evaluation of the functional response of *C. lividipennis* to *N. lugens* eggs indicated that the tested Cry1Ab rice had no significant effect under two possible scenarios, i.e., *C. lividipennis* reared on TN1 plants (BPH-susceptible, non-Bt rice) preyed on *N. lugens* eggs laid on Bt rice and non-Bt rice, and *C. lividipennis* reared on Bt rice or non-Bt rice preyed on *N. lugens* eggs laid on TN1 plants. To date, only a few studies have been conducted to assess the effects of Bt plants on predation of natural enemies. Tian *et al.* (2012) investigated the effect of Bt rice on predation of *N. lugens* by *P. pseudoannulata*, and found that Bt rice did not affect the predation in the field and the functional response in the lab. Those results reveal that Bt rice does not harm the biology of natural

enemies nor their pest control capability.

C. lividipennis plays a significant role in controlling *N. lugens*. However, its pest suppression potential often is impaired by applications of broad spectra insecticides, e.g. organophosphates (including Triazophos, Dichlorvos and Methamidophos) targeting stem borers in rice fields (Sun *et al.* 2008; Zhao *et al.* 2008; Wang *et al.* 2012; Yang *et al.* 2012). The decline of natural enemy populations caused by unjustifiable insecticide application is one reason for *N. lugens* outbreaks (Cheng and Zhu 2006). The safeness of Bt rice toward *C. lividipennis* in rice fields demonstrated by our and previous studies indicate that the effect of Bt rice on natural enemies is most likely minimum. Combined with the fact that Bt rice could reduce the use of insecticides against stem borers, Bt rice technology is compatible with IPM strategy and could serve as a viable tool for rice pest management.

4. CONCLUSION

Our studies indicated that the tested Cry1Ab rice does not adversely impact the biological character and functional response of *C. lividipennis* preying on *N. lugens* eggs. These results, together with other published literatures, demonstrate no adverse effects of Cry1Ab rice on *C. lividipennis*.

5. MATERIALS AND METHODS

5.1 Plant materials

A transgenic rice line KMD1 carrying the *cry1Ab* gene (Shu *et al.* 1998) was provided by Zhejiang University and used in this study. The untransformed parental commercial cultivar, Xiushui 11, was used as a control. The Cry1Ab rice and non-Bt rice plants at tillering stage were used to evaluate the development and reproduction of *C. lividipennis*. All plants were grown under natural light and ambient temperature in a screen-house.

5.2 Insects

A colony of *N. lugens* was collected from paddy fields at the Farm of China National Rice Research Institute in Fuyang City, Zhejiang Province, China (120.1 °E, 30.1 °N). *N. lugens* were maintained on a hopper susceptible variety “Taichung Native” (TN1). A colony of *C. lividipennis* was collected from the same fields and maintained with *N. lugens* on TN1. Both *N. lugens* and *C. lividipennis* were reared for two generations in the laboratory before used in the experiments.

5.3 Effect of Bt rice on the biological characters of *C. lividipennis*

To assess *C. lividipennis* nymphal development and adult emerge rate, three gravid female *N. lugens*

and three male *N. lugens* were confined with a potted Bt rice or non-Bt rice plant in a transparent plastic cylindrical cage (diameter 6 cm; height 40 cm) with a pair of mesh windows (diameter 4 cm). After 24 h, 10 newly hatched *C. lividipennis* were released into each cage. After 7 d, two more gravid female *N. lugens* were added into each cage to ensure enough eggs for *C. lividipennis*. The number of adults emerged were recorded daily and fresh weight was obtained. There were 15 replications for each treatment (Bt rice or non-Bt rice). The emerged adults were randomly selected for further biological observation and Cry1Ab protein detection described below.

Adult longevity and fecundity (number of eggs laid by each female) were evaluated by confining a pair of *C. lividipennis* adults (emerged from above experiment within 12 h) with a new potted plant of the same treatment that was pre-infested with three gravid female *N. lugens* and three male *N. lugens* for 24 h. The rice plants were changed weekly until all *C. lividipennis* adults died. The number of eggs laid by each *C. lividipennis* female was counted by dissecting each plant under a microscope. A total of 40 pairs were prepared for each treatment. The ones with *C. lividipennis* escaped or killed by accident were excluded.

Egg duration and hatching rate were accessed using 25 randomly selected rice plants of each treatment from the 40 setups mentioned above that were confined with a pair of *C. lividipennis* adults for 1 d only. Newly hatched *C. lividipennis* from those plants were recorded and removed daily until no more *C. lividipennis* hatching was observed. The number of un-hatched eggs was counted under the microscope.

All experiments were conducted at 27 ± 2 °C and 70 % - 80% RH under a photoperiod of 14:10 h (light: dark).

5.4 Cry1Ab protein detection in rice plants, *N. lugens* eggs and *C. lividipennis*

After *C. lividipennis* emergence, 20 adults from each treatment were confined to a potted Bt or non-Bt rice plant with a cage described above. Each of the plants was pre-infested with 10 gravid females and 10 males of *N. lugens* for 24 h. The *C. lividipennis* adults were allowed to feed for 5 d before collected for testing. Rice stem and *C. lividipennis* samples were collected with three replications of 20 mg each., *N. lugens* eggs were collected by dissecting the rice plants with three replications of 20 eggs each. All samples were stored at -70 °C until use.

To determine Cry1Ab protein levels in the samples, ELISA was carried out using the QualiPlate™ Kit for Cry1Ab/Cry1Ac (Enviroligix, Portland, ME). Prior to analysis, all insect samples were washed in phosphate-buffered saline with Tween-20 (PBST) buffer (provided in the kit) four times to remove

any Bt protein on the surface. Samples were homogenized and diluted at a rate of 1: 1000 (mg samples: μl PBST) for plants and a rate of 1: 20 (mg samples: μl PBST) for insects. The solutions were mixed for 1 min on a vortex mixer, centrifuged for 5 min at $12,000 \times g$, and loaded at $50 \mu\text{l}$ per test well. ELISA was performed according to the manufacturer's instructions. Spectrophotometric measurements were taken using a multi-detection microplate reader (Synergy HT, Bio-Tek, Winooski, VT) at 450 nm. A calibration curve was generated using purified Cry1Ab protein (Case Western Reserve University, Cleveland, OH) at concentrations of 0, 0.3125, 0.625, 1.25, 2.5 and 5 ng ml^{-1} .

5.5 Functional responses of *C. lividipennis*

Test 1: This test was designed to evaluate potential effects of *N. lugens* eggs laid on Bt rice on the functional response of *C. lividipennis*. One gravid female *N. lugens* was confined with a potted Bt rice or non-Bt rice plant with a transparent plastic cylindrical cage described above. After 48 h, the *N. lugens* was removed and the number of *N. lugens* eggs on the plant was counted under the microscope. Rice was placed into a glass tube (Diam 3.0 cm; Ht 25cm). Cotton wool with a 1% benzimidazole solution (Sigma, St. Louis, MO) was used to cover the rice root to retain moisture. One 2-d-old, mated female *C. lividipennis* that emerged from TN1 rice was transferred into each tube after 12 h starvation. The number of *N. lugens* eggs preyed by the *C. lividipennis* was counted after 24h. The used density ranges of *N. lugens* eggs on Bt or non-Bt rice were as follows: 0-10, 11-20, 21-30, 31-40, 41-50, ≥ 51 . Each range was replicated 4 times.

Test 2: To evaluate potential effects of *C. lividipennis* reared on Bt rice on its functional response to *N. lugens* eggs laid on TN1, this test was carried out as described above except that the *C. lividipennis* used was emerged from Bt rice or non-Bt rice with *N. lugens* eggs laid on TN1 rice as preys.

5.6 Data analysis

All biological parameter data were analyzed using the Student's *t*-test performed by DPS software (Tang *et al.* 2012). The functional response data were fitted to the Holling's "Type II": $N_a = aT_h N_0 / (1 + bN_0)$, where N_a is the number of *N. lugens* eggs preyed by *C. lividipennis*, N_0 is the number of *N. lugens* eggs before predation occurred, T_h is the duration of the test (24 h). The parameters a (instantaneous search rate) and b (time required to handle a prey) were calculated using PROC NLIN method by SAS v.9.1 (SAS Institute 2001), and those from the Bt rice treatment and non-Bt rice treatment were compared using the student's *t*-test. For all tests, $\alpha=0.05$.

Acknowledgements

We greatly appreciate Prof. Q. R. Shu (Zhejiang University, China) for providing the

transgenic rice used in this study, Mrs. J. Zhao (Hangzhou Normal University, China) for her technical assistance, and H. L. Collins (Cornell University, Ithaca, New York, USA) for editing the manuscript. Financial support was provided by the National Genetically Modified Organisms Breeding Major Projects (2011ZX08012-004) and Zhejiang Provincial Natural Science Foundation of China (LQ13C030003).

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1

2 **Table 1 Effect of Cry1Ab rice on life table parameters of *Cyrtorhinus lividipennis***

Parameters	Cry1Ab rice	Non-Bt isolate	Student's <i>t</i> -test
Survival rate (%)	75.3±18.1 (15)	61.3±21.3 (15)	<i>t</i> =1.94, <i>df</i> =28, <i>P</i> =0.06
Nymphal development duration (d)			
Female	9.4±0.9 (15)	9.7±0.8 (15)	<i>t</i> =2.05, <i>df</i> =28, <i>P</i> =0.06
Male	9.5±0.9 (15)	9.3±0.8 (15)	<i>t</i> =0.92, <i>df</i> =28, <i>P</i> =0.36
Adult fresh weight (mg)			
Female	0.90±0.12 (15)	0.88±0.15 (15)	<i>t</i> =0.75, <i>df</i> =28, <i>P</i> =0.46
Male	0.60±0.09 (15)	0.57±0.07 (15)	<i>t</i> =1.52, <i>df</i> =28, <i>P</i> =0.13
Adult longevity (d)			
Female	19.7±9.1 (35)	17.4±10.6 (36)	<i>t</i> =0.94, <i>df</i> =69, <i>P</i> =0.35
Male	26.3±16.1 (33)	32.8±13.9 (35)	<i>t</i> =1.78, <i>df</i> =66, <i>P</i> =0.08
Fecundity	177.6±129.3 (35)	181.8±147.1 (36)	<i>t</i> =0.12; <i>df</i> =69; <i>P</i> =0.90
Egg duration (d)	9.4±0.6 (25)	9.3±0.6 (25)	<i>t</i> =1.17; <i>df</i> =48; <i>P</i> =0.24
Hatching rate (%)	89.5±13.8 (25)	86.9±19.5 (25)	<i>t</i> =0.54, <i>df</i> =48, <i>P</i> =0.59

3 **Means ±SD (n), with Student's *t*-test analysis.**

4

Table 2 Parameters of type II functional response by *Cyrtorhinus lividipennis* to *Nilaparvata lugens* eggs

	Tested material	Parameters of Type II functional response		R ² (%)
		<i>a</i>	<i>b</i>	
Test 1	Cry1Ab rice	0.81 ±0.08	0.020 ±0.004	93.16 ±2.86
	Non-Bt isoline	0.65 ±0.06	0.016 ±0.002	97.34 ±0.52
Test 2	Cry1Ab rice	1.28 ±0.13	0.032 ±0.003	96.90 ±0.47
	Non-Bt isoline	1.10 ±0.15	0.028 ±0.002	97.27 ±0.87

Test 1: *C. lividipennis* reared on TN1 plants responses to *N. lugens* eggs laid on Cry1Ab rice or non-Bt isoline

Test 2: *C. lividipennis* reared on Cry1Ab rice or non-Bt isoline plants responses to *N. lugens* eggs laid on TN1 plants

a: Instantaneous search rate (day⁻¹)

b: Time required to handle a prey (day)

Parameter *a* or *b* was not significantly different between the Bt rice and non-Bt rice treatments within each test, based on Student's *t*-test ($P < 0.05$).

Figures

Fig. 1. Functional response of *Cyrtorhinus lividipennis* to *Nilaparvata lugens* eggs

A: Predation of *N. lugens* eggs laid on Cry1Ab rice or non-Bt rice by the *C. lividipennis* reared on TN1 plants; B: Predation of *N. lugens* eggs laid on TN1 plants by the *C. lividipennis* reared on Cry1Ab rice or non-Bt rice plants.

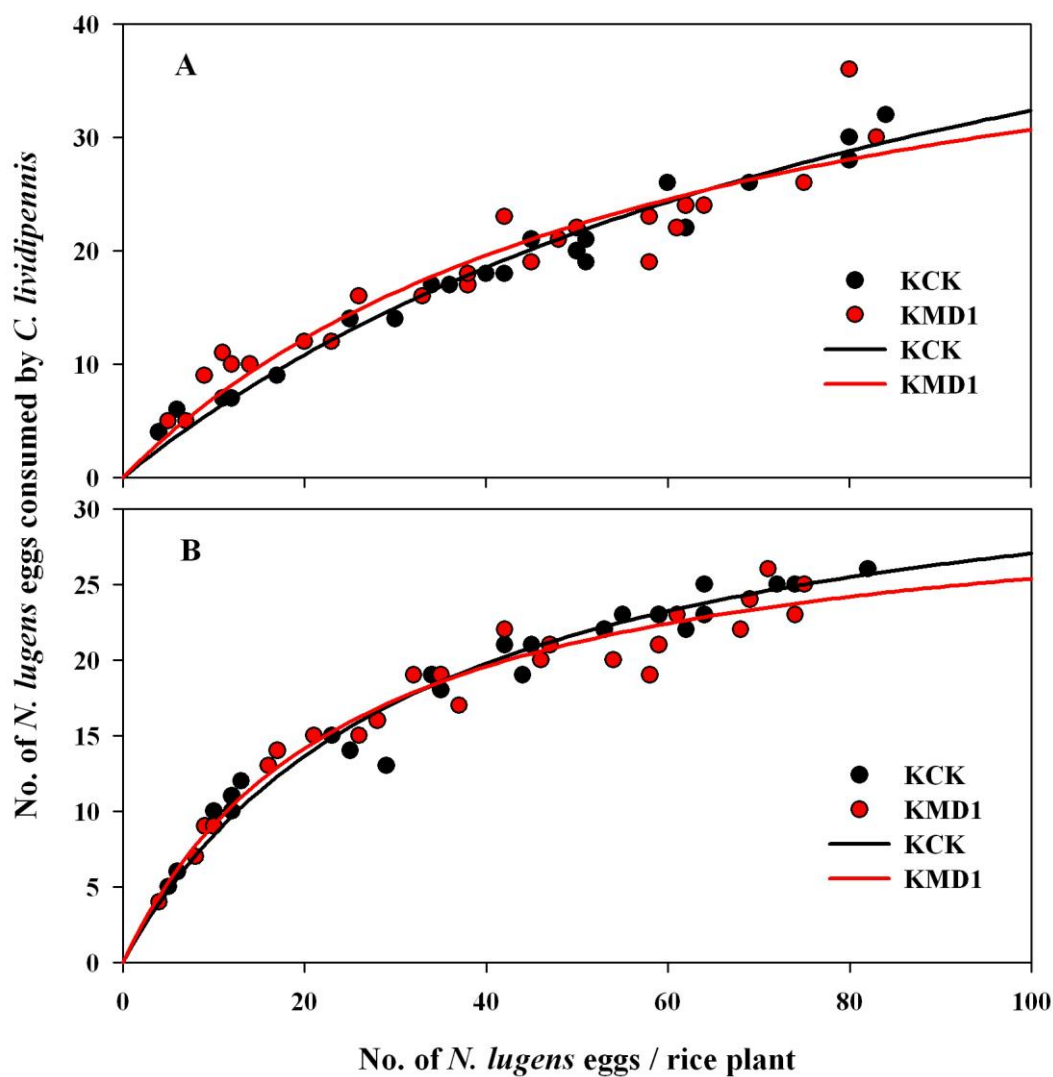


Fig. 1.