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The effects of triazophos applied to transgenic Bt rice on the nutritional indexes, *Nlvg* expression, and population growth of *Nilaparvata lugens* Stål under elevated CO_2

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ABSTRACT

The brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a typical pest in which population resurgence can be induced by insecticides. Warmer global temperatures, associated with anthropogenic climate change, are likely to have marked ecological effects on terrestrial ecosystems. However, the effects of elevated CO_2 (eCO_2) concentrations on the resurgence of *N. lugens* that have been treated with pesticides used for transgenic Bt rice cultivation are not fully understood. The present study investigated changes in the protein content, soluble sugar content, free amino acid level, vitellogenin (*Nlvg*) mRNA expression, and the population growth of *N. lugens* on transgenic Bt rice (TT51) following triazaophos foliar spray under conditions of eCO_2. The results showed that the protein content in the fat bodies and ovaries of *N. lugens* adult females in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than under ambient CO_2 (aCO_2) and was also higher than that in females feeding on the non-transgenic parent (MH63) under aCO_2 at different days after emergence (DAEs). The soluble sugar content and free amino level of adult females in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2 and was also higher than in MH63 under aCO_2 at 1 and 3 DAE. The *Nlvg* mRNA expression level of *N. lugens* adult females in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2 and was also higher than in MH63 under aCO_2 at 1 and 3 DAE. The population number of *N. lugens* in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2 and was also higher than in MH63 under aCO_2. The present findings provide important information for integrated pest management with transgenic varieties and a better understanding of the resurgence mechanism of *N. lugens* under eCO_2.

1. Introduction

The brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a classic example of a pest that can demonstrate insecticide-induced resurgence [1,2]. Pyrethroid (deltamethrin) and organophosphate (triazophos, methamidophos, paratihion and diazinon) insecticides are known to induce population resurgence of this species [3–6]. Physiological mechanisms for the pesticide-induced resurgence of *N. lugens* involve stimulation of fecundity [4,6–8]. Our previous studies have shown that pesticide applications result in changes to biochemical substances in rice plants [9], increased contents of crude fat and soluble sugars in nymphs and adults [6], elevated levels of ovary protein, RNA [10,11] and *Nlvg* mRNA expression level in *N. lugens* adult females [12–15].

Atmospheric carbon dioxide (CO_2) levels have risen steadily since the start of the industrial revolution, from 280 to 387 ppm today [16]. Current levels of atmospheric CO_2 are expected to double within the next 100 years [17]. A number of studies have examined how various ecosystems might respond to changing atmospheric levels of this gas [18–20]. Profound impacts of elevated CO_2 in terrestrial ecosystems [21,22], especially on the chemical composition and nutrient quality of plants, are expected [23,24]. Previous studies have mainly focused on changes in plant photosynthesis, growth, above-ground biomass, leaf area, yield and carbon:nitrogen (C:N) ration, particularly in C_3 plants [25–28]. These studies have shown that elevated CO_2 indirectly affected phloem-sucking and herbivorous insects, and the major influence of elevated CO_2 on insects is through a cascade effect when host plants alter their primary and secondary metabolites [29]. However, the effects of elevated CO_2 concentrations on nutritional indexes, population growth, and the *Nlvg* gene expression levels of *N. lugens* feeding on transgenic Bt rice plants following treatment with pesticides are not well understood.

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The objectives of this study were to evaluate (1) the effects of triazophos on the reproduction parameters of *N. lugens* feeding on transgenic Bt rice plants under eCO_2; (2) the changes in quantity of energy and nutrient substances in *N. lugens* on transgenic Bt rice plants treated with triazophos under eCO_2; and (3) the changes in population numbers of *N. lugens* on transgenic Bt rice plants treated with triazophos under eCO_2 conditions.

2. Materials and methods

2.1. Open-top chambers

The experiment was performed using eight octagonal, open-top chambers (OTCs), each measuring 4.2 m in diameter, located at the Observation Station of the Global Change Biology Group, Institute of Zoology, Chinese Academy of Science (CAS) in Xiaotangshan County, Beijing, China (40°11′ N, 116°24′ E). The atmospheric CO_2 concentration treatments were: (1) the current atmospheric CO_2 levels (375 μl/l) ("ambient CO_2", "aCO_2"), and (2) doubled the ambient CO_2 level (750 μl/l) ("elevated CO_2", "eCO_2"). Four OTCs were used for each concentration treatment. CO_2 concentrations were monitored from the rice seedling stage to the end of the experiment and were adjusted with an infrared CO_2 analyzer (Ventostat 8102; Telaire, Goleta, CA, USA) once every 20 min to maintain the CO_2 concentrations. The automatic-control system for adjusting the CO_2 concentration and the specifications for the OTC are described in detail in Chen et al. [30].

2.2. Rice varieties and culture

The transgenic Bt rice line (TT51) and the non-transgenic parental *indica* rice line Minghui 63 (MH63) were selected for the OTC evaluation. TT51 is a transgenic rice line expressing a *Bt* fusion gene derived from cry1Ab and cry1Ac under the control of the rice *actinI* promoter [31]. The transgenic Bt rice line was provided by Lin YongJun, National Key Laboratory of Crop Genetic Improvement, Wuhan, China. Seeds were sown outdoors in a standard rice-growing soil in cement tanks (height 60 cm, width 100 cm and length 200 cm). When the seedlings reached the six-leaf stage, they were transplanted into 16 cm diameter plastic pots with four hills per pot and three plants per hill and placed in OTCs. Each OTC contained 24 plastic pots (i.e., twelve plastic pots for MH63 and twelve plastic pots for TT51). The pots were randomly placed in each OTC and re-randomized daily to minimize position effects. From the six-leaf stage until the end of the experiment, pure CO_2 mixed with ambient air was supplied to each of the OTCs in the elevated CO_2 treatment group, and ambient air alone was supplied to the OTCs of the ambient CO_2 treatment group.

2.3. Insects culture and insecticide

In the experiments, the rice brown planthopper, *N. lugens* was obtained from a laboratory population maintained in a greenhouse under our standard conditions (26 ± 2 °C, with 70–80% humidity and a 16L:8D (light:dark photoperiod)) (size: height 250 cm, width 400 cm, length 500 cm) at an ecological laboratory at Yangzhou University, that were originally obtained from the China National Rice Research Institute (CNRRI; Hangzhou, China). Prior to the beginning of the experiments, the *N. lugens* colony was allowed to reproduce for two generations in cement tanks under natural conditions in Beijing. Technical triazophos (87% [AI]) was purchased from the Shenli Pesticide Co., Ltd., Ningguo, Anhui, China.

2.4. Treatment setup of triazophos spraying

Triazophos was dissolved in acetone. Ten percent of an emulsifier was added and diluted in a 40 ppm concentration with tap water based on previous results from a sublethal test (40 ppm triazophos treatment significantly induced a resurgence of *N. lugens*) [10,32]. Rice plants at the tillering stage were sprayed using a Jacto sprayer (Maquinas Agricolas Jacto S.A., Brazil) equipped with a cone nozzle (1 mm diameter orifice, pressure 45 psi, and flow rate 300 ml/min), to apply 40 ppm of triazophos in 100 ml of spray per pot. Control plants at the same stage were sprayed with the same amount of acetone and emulsifier. One hundred twenty third-instar nymphs were released per pot 24 h after spraying. After adults emerged, females were transferred to rice plants of the same growth period. Adult females were collected after 1 and 3 days, respectively. The protein content, soluble sugar content, free amino acid level, and *Nlvg* mRNA expression level of adult females were measured. Each treatment and control were replicated four times.

2.5. Extraction of protein from fat bodies and ovaries of adult females

Protein was extracted from fat bodies and ovaries using a method similar to that of Gong et al. [33]. Individual adult female *N. lugens* were dissected under a zoom-stereomicroscope (model XTL20, Beijing Tech Instrument Co., Ltd., Beijing, China) in a cooled petri dish. Firstly, the head and thorax of *N. lugens* adult female were scissored (size: 125 mm curved pointed scissors, PL8301, Pulun medical instrument Co., Ltd., Shanghai, China), then scissored along the back of adult female; secondly, ovaries were removed, then the fat body around ovaries and the fat body under the body wall were removed. The ovaries and fat bodies of females were removed and placed in separate, pre-weighed, ice-cold centrifuge tubes and then re-weighted using a Mettler-Toledo electronic balance (EC100 model; 1/10,000 *g* sensitivity). A proportional amount of NaCl solution (0.4 M NaCl:1 M PMSF; vol:vol at a ratio of 20 ml NaCl solution to 1 *g* ovaries or fat bodies) was added to the tube, homogenized on ice, and centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatant was collected after filtering the upper fat layer with glass fibers, placed at 4 °C overnight after adding ddH_2O (1 supernatant:10 ddH_2O; vol:vol), and centrifuged again at 5000 rpm at 4 °C for 20 min. The protein sediment was dissolved with 1.5 ml pre-cooled 0.4 M NaCl solution after removing the supernatant.

2.6. Measurement of protein content

The procedure described by Li and Yu [34] was used to measure protein content using Coomassie Brilliant Blue R 250 (Shanghai Chemical Agent Co., Ltd., Shanghai, China). A standard curve was established based on a standard protein (bovine serum albumin, Shanghai Biochemistry Research Institute, Shanghai, China). The supernatant obtained from the procedure described above (1 ml) and 5 ml of Coomassie Brilliant Blue R 250 were combined in a test tube and shaken. The absorbance at 595 nm was determined in a UV755B spectrometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China) after 5 min. The protein content in the sample solution was calculated according to the standard curve.

2.7. Determination of soluble sugar

Soluble sugars were determined based on the anthrone reagent method [35]. To begin, 1 mg of adult females was homogenized with 1 ml of 30% KOH, then heated in a boiling water bath for 20 min, and cooled down to room temperature. 3 ml of absolute alcohol (99.7%) was added, and the mixture was centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant was collected after filtering the

upper fat layer with glass fibers. 2 ml of the supernatant was placed into a 10 ml test tube to which 3 ml of 0.6 mol/l HCl, heated in a boiling water bath for 2 h, cooled down for 15 min, 1 ml anthrone reagent was added. The test tube was placed again in a water bath, boiled for 15 min, and cooled down to room temperature. The absorbency at 620 nm was measured using a UV755B spectrometer (The 3rd Analytical Instrument Company of Shanghai, Shanghai, China). A standard curve was drawn with sucrose.

2.8. Determination of free amino acid

Free amino acids were determined based on the method of ninhydrin [35]. First, 1 g of adult females was homogenized with 5 ml of 10% acetic acid. The solution was filtered, and the filtered solution was transferred to a 100 ml volumetric flask, and mixed with distilled water to achieve a total volume of 100 ml and then filtered evenly shaking. 1 ml of extraction solution was transferred to a 25 ml volumetric flask, and 3.5 ml of ninhydrin buffer developing solution was added and thoroughly shaken. Then, 0.1 ml of 0.1% ascorbic acid solution was added, and the mixture was thoroughly shaken. The mixture was heated in a boiling water bath for 20 min, then removed and cooled rapidly. Following this process, 10 ml of 80% ethanol was added, and then water was added to achieve a total volume of 25 ml and thoroughly shaken. Finally, 6 ml of mixture solution was absorbed to measure its absorbency. The absorbency at 570 nm was measured using a UV755B spectrometer (The 3rd Analytical Instrument Company of Shanghai, Shanghai, China). A standard curve was drawn with glutamic acid.

2.9. Population growth experiment

A population growth experiment was conducted according to the experimental design of Wu et al. [36]. Rice plants at the tillering stage were sprayed using a Jacto sprayer (Maquinas Agricolas Jacto S.A., Brazil) equipped with a cone nozzle (1-mm diameter orifice, pressure 45 psi, and flow rate 300 ml/min), applying 40 ppm of triazophos in 100 ml of spray per pot. Control plants at the same growth stage were sprayed with the same amount of acetone and emulsifier. Per pot treated and control rice plants at the tillering stage were removed from OTC, and sprayed 40 ppm of triazophos and the same amount of acetone and emulsifier, respectively, then stuck on the guide, and moved into the OTC. Seven days after insecticide application, eight third-instar nymphs were released in each pot, using nylon cylindrical cages (20 cm diameter × 80 cm height; screen size: 80-mesh). All treatments and controls were replicated four times. When the 3rd instar nymphs of the next generation appeared in the cages, all nymphs (1st, 2nd and 3rd instars) and unhatched eggs were counted. The population growth index (PGI) was expressed by the ratio of N_1/N_0, which was calculated by dividing the total number (N_1) of nymphs and eggs by the number of nymphs released ($N_0 = 8$).

2.10. Total RNA isolation and cDNA preparation

Total RNA was isolated from five of the emerging adult females, which were collected as described above (section 2.4), using an SV Total Isolation System Kit (model Z3100, Promega Corporation, Madison, WI, USA). First-strand cDNAs were synthesized according to the instructions supplied with the PrimeScript RT reagent Kit (TaKaRa Biotechnology (Dalian) Co., Ltd). First-strand cDNA was synthesized in a 10 μl total reaction volume containing 0.5 μg of total RNA, 0.5 μl of PrimeScript RT enzyme mix I, 0.5 μl of Oligo dT primer (50 μM), 2 μl of random hexamers (100 μM), 2 μl 5 × PrimeScript Buffer (for real time-PCR) and RNase-free dH_2O to a final volume of 10 μl. The cDNA was reverse transcribed using the following cycling regime: 37 °C for 15 min, 85 °C for 5 s and 4 °C for 5 min.

2.11. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The level of *Nlvg* mRNA expression in *N. lugens* adult females was determined by qRT-PCR using a Light Cycler 480 system (Roche Diagnostics, Indianapolis, IN, USA) and a SYBR Premix Ex Taq II Kit (TaKaRa Biotechnology Co., Ltd, Japan). qRT-PCR was performed in a 20-μl total reaction volume containing 0.1 μg of total RNA, 0.8 μl primer mix containing 10 μM each of the forward and reverse gene-specific primers, 0.4 μl ROX Reference Dye II (50×), 2 μl of cDNA, 10.0 μl SYBR Premix EX Taq II and 6.0 μl of H_2O. Non-template reactions (NTC) (replacing total RNA with H_2O) and minus reverse transcriptase controls (replacing the PrimeScript RT Enzyme Mix with H_2O) were used as negative controls. qRT-PCR was performed with the following cycling regime: an initial incubation at 55 °C for 5 min and 95 °C for 15 s, followed by 40 cycles of 95 °C for 15 s, 58 °C for 40 s and 72 °C for 15 s. β-actin (EU179846) was used as an internal control. *Nlvg* (AB353856) mRNA levels were quantified in relation to constitutive *N. lugens* β-actin expression. The mean values and standard errors for each time point were obtained from the average of three independent sample sets. The following gene-specific primers for Vg and β-actin were used: *Vg*-F: GTGGCTCGTTCAAGGTTATGG; *Vg-R*:GCAATCTCTGGGTGCTGTTG; *β*-F:TGGACTTCGAGCAGGAAATGG; and *β-R*: ACGTCGCACTTCA GATCGAG. The results were standardized to the constitutive *N. lugens* β-actin expression level. A relative quantitative method ($\triangle\triangle C_t$) was used to evaluate quantitative variation [37].

2.12. Statistical analysis

Data were evaluated for normality and homogeneity of variance using Bartlett's test [38]. Based on these evaluations, no transformations were needed. The protein content, soluble sugar content, free amino acid content, *Nlvg* mRNA expression level, and population growth were analyzed using an analysis of variance (ANOVA) with three factors (CO_2 concentration, rice variety, insecticide concentration). Multiple comparisons of the means were conducted using Fisher's Protected Least Significant Difference (PLSD) test. All analyses were conducted using the data processing system (DPS) of Tang and Feng [39].

3. Results

3.1. Effect of triazophos on the protein content in the fat bodies and ovaries of N. lugens adult females under eCO_2

The three-way ANOVA of the data (Fig. 1), showed that insecticide concentration, rice variety and CO_2 concentrations significantly influenced the protein content of fat bodies in the adult females (Table 1). The protein content of fat bodies significantly increased with 40 ppm triazophos treatment for both MH63 and TT51 varieties at different DAEs, under both aCO_2 or eCO_2 (Fig. 1A–C). For the two concentrations of CO_2, the grand mean (means of main effect) of the protein content of fat bodies treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2, increasing by 45.4% and 47.4%, at 1 and 3 DAE, respectively. For the two rice varieties, the grand mean of the protein content of fat bodies in TT51 treated with 40 ppm triazophos was significantly higher than that in MH63, increasing by 38.9% and 29.6%, at 1 and 3 DAE, respectively. Multiple comparisons of the means indicated that the protein content was significantly higher in the fat bodies treated with 40 ppm triazophos for both MH63 and TT51 than in the untreated controls; the protein content was significantly higher in the fat bodies for both MH63 and TT51 under eCO_2 than in those under aCO_2.

The three-way ANOVA of the data (Fig. 1) showed that insecticide concentration, rice variety and CO_2 concentration significantly

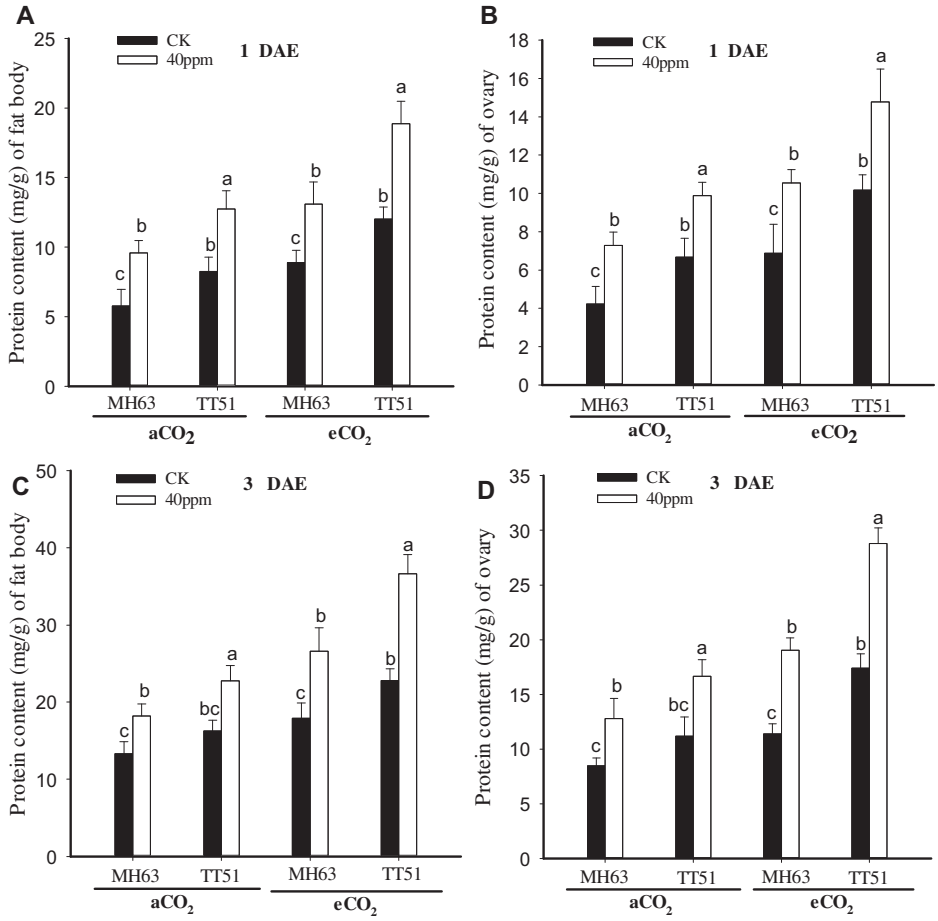

Fig. 1. Changes in the protein content of fat bodies and ovaries treated with 40 ppm triazophos at the 3rd instar for the tillering stage of Minghui 63 (MH63) and TT51 (Transgenic) under aCO_2 and eCO_2 environments. Bars with different letters (two groups were compared between MH63 and TT51 under aCO_2 or under eCO_2) are significantly different at the 5% level. Each treatment and control was replicated four times. DAE is days after emergence.

influenced the protein content of ovaries (Table 1). Similarly, the protein content of ovaries significantly increased with the 40 ppm triazophos treatment for both MH63 and TT51 at different DAEs, under both aCO_2 or eCO_2 (Fig. 1B–D). For the two concentrations of CO_2, the grand mean of the protein content of ovaries treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2, increasing by 50.9% and 56.2% at 1 and 3 DAE, respectively. For the two rice varieties, the grand mean of the protein content of ovaries in TT51 treated with 40 ppm triazophos was significantly higher than in MH63, increasing by 43.5% and 43.2% at different 1 and 3 DAEs, respectively. Multiple comparisons of the means indicated that the protein content was significantly higher in ovaries treated with 40 ppm triazophos for both MH63 and TT51 than in the untreated controls; the protein content was significantly higher in the ovaries for both MH63 and TT51 under eCO_2 than in those under aCO_2.

Table 1
Analysis of variance of protein content, soluble sugar and free amino acid content, and *Nlvg* mRNA expression level data shown in Figs. 1–3.

DAE	Source of variance	Df	Fat body		Ovary		Soluble sugar		Amino acid		*Nlvg* mRNA	
			F-value	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
1	CO_2 concentration (A)	1	100.7	0.0001	90.4	0.0001	234.7	0.0001	199.6	0.0001	44.1	0.0001
	Rice varieties (B)	1	78.2	0.0001	69.9	0.0001	547.9	0.0001	115.9	0.0001	72.8	0.0001
	Insecticide concentration (C)	1	138.4	0.0001	93.5	0.0001	1113.2	0.0001	234.1	0.0001	1670.6	0.0001
	A × B	1	3.9	0.057	2.7	0.116	8.2	0.0086	6.5	0.0177	1.3	0.273
	A × C	1	2.8	0.1081	1.5	0.1962	10.1	0.004	6.4	0.0187	42.3	0.0001
	B × C	1	4.2	0.0523	0.5	0.4678	131.1	0.0001	1.4	0.2564	52.9	0.0001
	A × B × C	1	1.4	0.2471	0.3	0.5943	1.1	0.308	6.9	0.0145	0.19	0.0662
3	CO_2 concentration(A)	1	137.9	0.0001	197.7	0.0001	270.9	0.0001	533.7	0.0001	124.5	0.0001
	Rice varieties (B)	1	62.3	0.0001	129.9	0.0001	444.1	0.0001	121.4	0.0001	237.6	0.0001
	Insecticide concentration (C)	1	142.5	0.0001	215.9	0.0001	848.6	0.0001	372.5	0.0001	4418.2	0.0001
	A × B	1	6.8	0.0158	22.1	0.0001	1.7	0.2079	20.5	0.0001	9.9	0.0043
	A × C	1	15.4	0.0006	22.3	0.0071	10.5	0.0035	47.2	0.0001	132.9	0.0001
	B × C	1	6.2	0.0258	6.2	0.205	87.6	0.0001	26.8	0.0001	155.5	0.0001
	A × B × C	1	1.7	0.2238	1.7	0.2034	3.3	0.0821	4.9	0.037	4.9	0.0366

3.2. Effect of triazophos on soluble sugar content and free amino acid content of adult females under eCO_2

The three-way ANOVA of the data (Fig. 2) showed that insecticide concentration, rice variety and CO_2 concentration significantly influenced the soluble sugar content of adult females (Table 1). The soluble sugar content of adult females increased significantly with the 40 ppm triazophos treatment for both MH63 and TT51 at different DAEs, under both aCO_2 or eCO_2 (Fig. 2A–C). For the two concentrations of CO_2, the grand mean of soluble sugar content of adult females treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2, increasing by 16.9% and 20.6% at 1 and 3 DAE, respectively. For the two rice varieties, the grand mean of the soluble content of adult females in TT51 treated with 40 ppm triazophos was significantly higher than in MH63, increasing by 27.0% and 29.9%, at 1 and 3 DAE, respectively. Multiple comparisons of the means indicated that the soluble sugar content was significantly higher in the adult females treated with 40 ppm triazophos for both MH63 and TT51 than in untreated controls; the soluble sugar content was significantly higher in the adult females for both MH63 and TT51 under eCO_2 than under aCO_2.

The three-way ANOVA of the data (Fig. 2) showed that insecticide concentration, rice variety and CO_2 concentration significantly influenced the soluble sugar content of adult females (Table 1). The free amino acid content of adult females significantly increased with 40 ppm triazophos treatment for both MH63 and TT51 at different DAEs, under both aCO_2 or eCO_2 (Fig. 2B–D). For the two concentrations of CO_2, the grand mean of free amino acid content of adult females treated with 40 ppm triazophos under eCO_2 was significantly higher than that of adult females under aCO_2, increasing by 30.6% and 41.9% at 1 and 3 DAE, respectively. For the two rice varieties, the grand mean of the free amino acid of adult females in TT51 treated with 40 ppm triazophos was significantly higher than in MH63, increasing by 22.5% and 27.9% at 1 and 3 DAE, respectively. Multiple comparisons of the means indicated that the free amino acid content was significantly higher in the adult females treated with 40 ppm triazophos for both MH63 and TT51 than in untreated controls; the free amino acid content was significantly higher in the adult females for both MH63 and TT51 under eCO_2 than in those under aCO_2.

3.3. Effect of triazophos on Nlvg mRNA express level of adult females under eCO_2

The three-way ANOVA of the data (Fig. 3) showed that insecticide concentration, rice variety and CO_2 concentration significantly influenced the *Nlvg* mRNA expression level of adult females (Table 1). The *Nlvg* mRNA of adult females significantly increased with the 40 ppm triazophos treatments for both MH63 and TT51 at different DAEs, under both aCO_2 or eCO_2 (Fig. 3). For the two concentrations

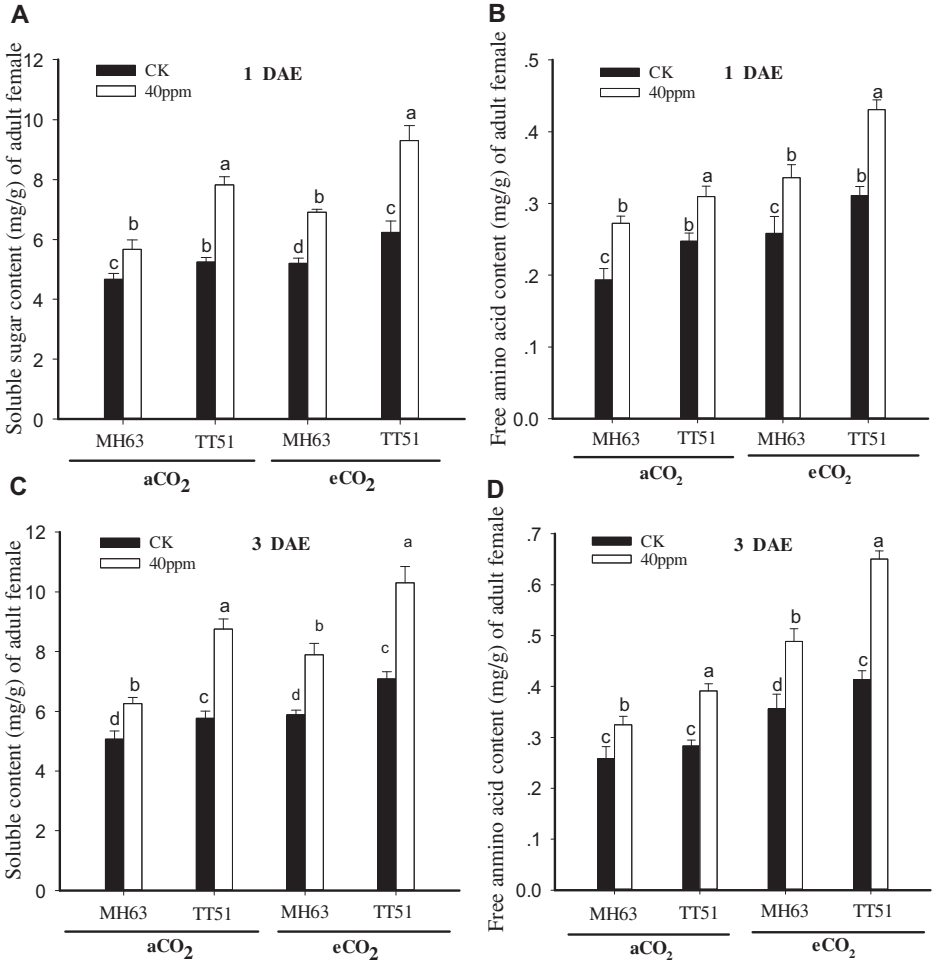

Fig. 2. Changes in the soluble sugar content (left) and free amino acid content (right) of adult females treated with 40 ppm triazophos at the 3rd instar for the tillering stage of Minghui 63(MH63) and TT51 (Transgenic) under aCO_2 and eCO_2 environments. Bars with different letters (two groups were compared between MH63 and TT51 under aCO_2 or under eCO_2) are significantly different at the 5% level. Each treatment and control was replicated four times. DAE is days after emergence.

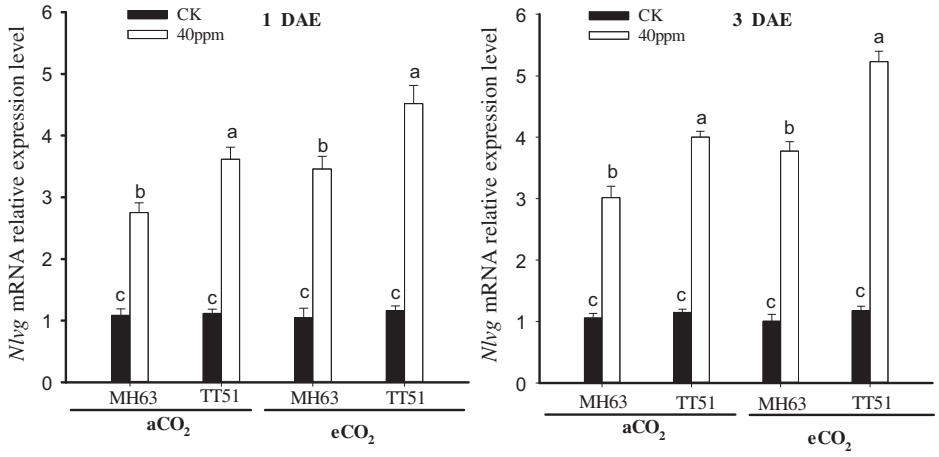

Fig. 3. Changes in the *Nlvg* mRNA expression level of adult females treated with 40 ppm triazophos at the 3rd instar for the tillering stage of Minghui 63(MH63) and TT51 (Transgenic) under aCO_2 and eCO_2 environments. All values were normalized relative to β-actin transcript levels. Bars with different letters (two groups were compared between MH63 and TT51 under aCO_2 or under eCO_2) are significantly different at the 5% level. Each treatment and control were replicated four times. DAE is days after emergence.

of CO_2, the grand mean of the *Nlvg* mRNA expression level of adult females treated with 40 ppm triazophos under eCO_2 was significantly higher than under aCO_2, increasing by 15.8% and 17.5% at 1 and 3 DAE, respectively. For the two rice varieties, the grand mean of the *Nlvg* mRNA expression level of adult females in TT51 treated with 40 ppm triazophos was significantly higher than in MH63, increasing by 19.9% and 23.3% at 1 and 3 DAE, respectively. Multiple comparisons of the means indicated that the *Nlvg* mRNA expression level was significantly higher in the adult females treated with 40 ppm triazophos for both MH63 and TT51 than in untreated controls; the *Nlvg* mRNA expression level was significantly higher in the adult females for both MH63 and TT51 under eCO_2 than in those under aCO_2.

3.4. *Effect of triazophos on the population growth of* N. lugens *under* eCO_2

The population number of *N. lugens* on MH63 or TT51 treated with 40 ppm triazophos was significantly higher than that of the control under aCO_2, increasing by 30.4% or 40.8%, respectively (Table 2). Similarly, the population number of *N. lugens* in MH63 or TT51 treated with 40 ppm triazophos was significantly higher than that of the control under eCO_2, increasing by 39.6% or 45.9%, respectively (Table 2). ANOVA of the data showed that insecticide concentration, rice variety and CO_2 concentration significantly influenced the population numbers of *N. lugens* under aCO_2 or eCO_2 (Table 2). For the two concentrations of CO_2, the grand mean of population numbers of *N. lugens* treated with 40 ppm triazophos under eCO_2 was significantly higher than those under aCO_2, increasing by 36.6% ($F = 12.4$, $df = 1, 31$, $P = 0.0018$). For the two rice varieties, the grand mean of population numbers of *N. lugens* in TT51 treated with 40 ppm triazophos was significantly higher than that on MH63, increasing by 47.1% ($F = 8.1$, $df = 1,31$, $P = 0.0088$). Multiple comparisons of the means indicated that the population numbers of *N. lugens* were significantly higher when treated with 40 ppm triazophos for both MH63 and TT51 than that of untreated controls; the population numbers for both MH63 and TT51 under eCO_2 were significantly higher than in those under aCO_2.

4. Discussion

Previous studies have shown that elevated CO_2 could lead to significant physiological and biochemical alterations of C_3 plants [27,40–42]. Elevated CO_2 did not directly affect phloem-sucking insects, but changes in the biochemical and nutrient substances of host plants insects were indirectly affected [43,44]. Our findings showed that the protein content in the fat bodies and ovaries of *N. lugens* adult females in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than for those in MH63 under aCO_2 (Fig. 1). Therefore, for TT51, the foliar spray of triazophos promoted the synthesis of protein in the ovaries and fat bodies of *N. lugens* adult females under eCO_2 conditions. Pesticides stimulate the reproduction of pests by increasing the protein and RNA content in the ovaries and fat bodies of adult females [10,32,45]. Physiologically, the fecundity of adult females is primarily regulated by the synthesis of vitellin (Vt) and vitellogenin (Vg), and Vg

Table 2
Changes in *N. lugens* population growth of 3rd instar treated with 40 ppm triazophos at the tillering stage of Minghui 63 (MH63) and TT51 (Transgenic) under aCO_2 and eCO_2 environments.

CO_2 concentration(ppm)	Rice varieties	Insecticide concentration(ppm)	Average number N_1*	N_1/N_0	Increase N_1/N_0 over control (%)
Ambient CO_2 (375 ppm)	MH63	0	980.0 ± 430.5b	122.5	
		40	1407.3 ± 464.6ab	175.9	30.40%
	TT51	0	1270.5 ± 560.0ab	158.8	
		40	2146.8 ± 668.9a	268.4	40.81%
Elevated CO_2 (750 ppm)	MH63	0	1383.5 ± 434.8b	172.9	
		40	2292.0 ± 592.3ab	286.5	39.63%
	TT51	0	1707.5 ± 552.2b	213.4	
		40	3153.3 ± 637.5a	394.2	45.85%

* Means ± SE of four replicates. Means within columns followed by different letters (two groups were compared between MH63 and TT51 under aCO_2 or under eCO_2) are significantly different at the 5% level ($P < 0.05$, PLSD test).

synthesis is also regulated by juvenile hormone (JH) in most insects [46,47]. The present findings showed that the *Nlvg* mRNA expression levels of *N. lugens* adult females in the TT51 variety treated with triazophos under eCO_2 were significantly higher than those under aCO_2 (Fig. 3). Previous studies have shown that some insecticides induced changes in *Nlvg* mRNA expression levels and promoted protein synthesis in the fat bodies and ovaries of *N. lugens* adult females, and stimulated fecundity of *N. lugens* adult females [12,13]. However, two insecticides (chlorantraniliprole and indoxacarb) suppressed *Nlvg* mRNA expression and the number of eggs oviposited by *N. lugens* females [14,15]. These findings indicated that *Nlvg* mRNA expression level was associated with the fecundity of *N. lugens* adult females.

Our findings showed that the soluble sugar content of *N. lugens* adult females in TT51 treated with 40 ppm triazophos under eCO_2 was significantly higher than that of females in MH63 under aCO_2 (Fig. 2). Ge et al. [48] reported that the soluble sugar content of TT51 treated with triazophos under eCO_2 was significantly higher than that of MH63 under aCO_2. Therefore, *N. lugens* may consume more phloem sap of TT51 to acquire sucrose (energy substances) requirements. Phloem-feeding insects, such as *N. lugens*, have access to a carbon rich diet, the main constituent of which, in many plant species, including rice, is sucrose [49,50]. Sucrose is present in plant phloem sap at high concentrations, often in the range of 0.5 M–1 M and is responsible for a high osmotic pressure, often up to five times that of the feeding Hemipteran's body fluids [51]. Yin et al. [6] reported that crude fat and soluble sugar contents in nymphs and adults developed from nymphal feeding on rice plants treated with insecticides was significantly increased compared with controls, indicating that insecticide-treated plants are suitable for *N. lugens* feeding. Foliar insecticide applications result in the accumulation of energy substances (lipids and soluble sugars) for *N. lugens* migration. Lipids and sugars are not only necessary substances for the growth, development, and movement (flight) of insects, but are also important components of insect yolk [52]. These two substances are also associated with insect reproduction. One of the main mechanisms of pest resurgence is increased reproduction caused by pesticides [4,7]. Zhao et al. [53] reported the flight capacity of adults *N. lugens*, developed from nymphs feeding on rice plants treated with insecticides (including triazophos), was enhanced, indicating that treated rice plants supply more energy for *N. lugens* flight. Therefore, the treatment of TT51 with triazophos under eCO_2 might be beneficial to the feeding and flight of *N. lugnes*.

Previous studies have shown that the free amino acid level of TT51 treated with triazophos under eCO_2 was significantly lower than that of MH63 under aCO_2 [48]. However, the present findings show that the free amino acid level of adult females in TT51 treated with triazophos under eCO_2 was significantly higher than those in MH63 under aCO_2 (Fig. 2). Therefore, *N. lugens* may consume more TT51 phloem sap to meet their nutritional (including amino acid) requirements. The increase of free amino acids or decline of C/N is an important factor for stimulating *N. lugens* feeding [54]. Rice plants treated with jingganmycin had significant effects on the promotion of *N. lugens* feeding [36]. Sun et al. [55] reported that lower amounts of amino acids were found in cotton phloem sap under eCO_2 than under aCO_2 levels. Higher amounts of free amino acids were found in *Aphis gossypii* fed on cotton grown under eCO_2 than those fed on cotton grown under aCO_2. These findings indicate that *A. gossypii* will consume more phloem sap to meet their nutritional requirements in an eCO_2 environment, which will balance amino acid content in the plants. Some specific amino acids may also play important roles in the interaction of rice plants and *N. lugens*. Effective amino acids tend to reduce the number of attempts to probe and increase honeydew excretion as a result of the promotion of sustained sucking, while others exert a marked inhibitory effect on sucking in *N. lugens* [56].

The present studies show that the population numbers of *N. lugens* for TT51 treated with triazophos under eCO_2 were significantly higher than for MH63 under aCO_2 (Table 2). However, Chen et al. [57] reported that the fecundity of *N. lugens* on Bt rice (CrylAb) under aCO_2 was significantly decreased in every generation, compared with the non-Bt rice in a field investigation, and did not result in an outbreak of its non-target herbivore (*N. lugens*). The single transgenic (Cry1Ab) Bt rice showed better performance in controlling the non-target planthopper *Sogatella furcifera* compared with the fused transgenic (Cry1Ab/Cry1Ac) Bt rice under eCO_2 and temperature [58]. Previous studies have shown that the oxalic acid and flavonoid contents of TT51 treated with triazophos under eCO_2 were significantly lower than those of MH63 under aCO_2, and also decreased the resistance of TT51 to *N. lugens* [48]. Therefore, for TT51, triazophos foliar spray under eCO_2 might be beneficial to the population growth of *N. lugens*. Pesticides may affect target insects indirectly, though altering nutritional and other biochemical aspects of host plants, and even lead to the resurgence of the target pests [59–61]. Some insecticides may increase susceptibility of plants to insects. Jingganmycin had significant effects on plant susceptibility to the insects [36]. Gao and Ma [62] reported that deltamethrin and parathion-methyl make rice plants susceptible to *N. lugens*. Some insecticide treatments result in 100% hopperburn of plants, whereas untreated plots suffer only minor or no damage [63].

The present results showed that *N. lugens* for TT51 treated with triazophos under eCO_2 had markedly up-regulated *Nlvg* mRNA expression levels, the nutritional indexes of *N. lugens* adult female significantly varied feeding on TT51 treated with triazophos in an eCO_2 environment, and the population numbers of *N. lugens* for TT51 treated with triazophos under eCO_2 significantly increased. However, except for the above-mentioned mechanisms, the overall resurgence mechanism of *N. lugens* in transgenic Bt rice plants treated with insecticides under eCO_2 needs further investigation.

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