Sublethal Effects of Bifenazate on Biological Traits and Enzymatic properties in the Panonychus citri (Acari: Tetranychidae)

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Abstract

Panonychus citri, is a major citrus pest with a global distribution. But in Pest Management, the extensive use of insecticides has resulted in insecticide tolerance. Bifenazate, a novel acaricides with high biological activity against red mites, such as the *T.urticae*, *P. citri.* However, its sublethal effects on *P. citri* have not been reported. Therefore, in present study, *P. citri* exposed to sublethal concentration of bifenazate shows high biological activity, and causes great changes of population parameters and enzymatic activity. Life table technology was used to demonstrate sublethal effects on the growth and reproduction of *P. citri.* The results showed that the developmental duration of immature stage used sublethal concentration of bifenazate (LC_{10}, LC_{30}) were no difference than control. Nevertheless, compared with control group, the female adult duration, the fecundity and longevity were significantly decrease, but the pre-oviposition period was longer; In addition, the net reproductive rate (R_0), the mean generation(T) were significantly decreased compared to those of the control, but the intrinsic rate of increase(r), the finite rate(λ) were decreased in LC_{30} group, however, the doubling time was increased with the increasing concentrations of bifenazate. Furthermore, Enzymatic tests showed that CAT, POD, and CarE activities were higher in LC_{10} and LC_{30} treatments than control. The SOD and GST activities were lower in LC_{30} treatment than control and LC_{10} treatment. Moreover, the CYP450 activity was decreased with the increasing concentrations of bifenazate. Based on these results, the sublethal concentration of bifenazate may have adverse effects on population growth of *P. citri*. This result provides certain significant guidance for the Pest Management. As well as that, further studies should be done to explore resistance mechanisms of *P. citri* exposure to a sublethal concentrations of bifenazate.

1. Introduction

Panonychus citri (Acarina:Tetranychidae) is a spider mite with worldwide distribution and causes large economic losses in many countries [1, 2]. The *P.citrus* has a stabbing mouthpiece and feeds on the juice from the leaves, shoots, flower buds, and fruits of Citrus[2, 3].Currently, the main strategy for *P.citrus* control remains chemical control, however it has developed strong tolerance for many acaricides, such as pyridaben, fenpyroximate [4, 5]. Therefore, it is necessary to select more effective insecticides for controlling this pest.

Bifenazate is a novel acaricide developed in recent years, which is toxic to leaf mites at all life stages[6]. At present, it is used to control spider mites on a variety of crops, including fruits and ornamental plants[7]. Additional, bifenazate has low toxicity to mammals and aquatic organisms; It has rapid knockdown and no cross resistance with other acaricides. These properties make bifenazate an ideal insecticide for spider mites control[6].

Insecticides are not only directly toxic to insects, but also affect population dynamics by affecting life table parameters of insects[8].After insecticide application in the field, pests may shows different biological and ecological traits as they are exposed to different doses of insecticide[9]. For example, cyantraniliprole at a low lethal concentration (LC_{30}) significantly inhibited fecundity in *Helicoverpa*

Assa[10]. However, *T.urticae* fecundity has been stimulated after exposure to spinetoram at sublethal concentrations (LC_{10} and LC_{20})[11]. Therefore, in Integrated Pest Management (IPM), it has a significant influence on the sublethal effects of insecticides.

The study is to comprehensively evaluate sublethal effects of bifenazate on *P. citri*, including its developmental time, fecundity, enzymatic activities. Our study may help in understanding the biological response of *P. citri* after bifenazate exposure and provide the basis for further research of resistance mechanisms of *P. citri* exposure to a sublethal concentrations of bifenazate.

2. Materials And Methods

2.1. Mite and pesticide

The laboratory strains of *P.citri* were collected from the citrus orchard in Wuning County, Jiujiang City, Jiangxi Province, China, In September 2019. The population was reared on the leaf disc of *Aurantii Fructus* in climate-controlled chamber, under the conditions of 26 ± 1°C, relative humidity 70%±10%, photoperiod 16 hours: 8 hours (L:D). Place on a wet sponge in a Petri dish (15 cm in diameter), cotton slivers were placed around each leaf to prevent mites from escaping, without exposure to insecticide.Bifenazate ($C_{17}H_{20}N_2O_3$; ≥99 % Purity) was provided by accustandard (New Haven, USA).All the studies involving plants (Aurantii Fructus leaves) have been carried out in accordance with relevant institutional, national or international guidelines.

2.2. Bioassay

Bioassay of female *P.citri* was performed according to the modified leaf dish dipping method of Ken and Yamamoto [12].The newly emerged third instar female adult mites were transferred to the leaves of *Aurantii Fructus* by small brush. 45 individuals were selected from each leaf. Wet cotton was put around the leaves with mites to prevent mites from escaping. The mites were placed in climate-controlled chamber. After 4 hours, the mites were examined by microscope, the dead and inactive individuals were removed. Then, use tweezers to immerse the leaves with mites in different concentrations of acaricide solution for 5 s, and take out the excess liquid and quickly suck it up with absorbent paper. Leaves with female adult mites soaked in 0.1% ethyl acetate solution were set as control treatment. Put the treated leaves into the prepared leaf dish and put them into the climate control chamber. Each treatment was repeated in 4 groups. After 24 hours, the mortality was recorded under microscope. If their foot doesn't move, it will be regarded as death. If the mortality of control group is less than 10%, it will be regarded as effective experiment.

2.3. Sublethal concentration of bifenazate effects on the life-history traits of *P. citri*

In this study, leaf dipping method and leaf dish feeding method were used, and the feeding table was made as shown in the Fig. 1. The third instar female adult mites were selected and placed on each leaf dish. After 4 hours, the leaves and female adult mites were soaked with bifenazate (LC_{10}, LC_{30}) by leaf dish dipping method. The leaves with female adult mites were soaked with 0.1% ethyl acetate solution as

control. Then they were raised in climate-controlled chamber. After 24 hours, the survivors were selected to the fresh rearing platform to continue feeding, and the same number of male adult mites were selected at the same time. After mating for 12 hours, 100 eggs were taken from each treatment for single feeding. After the development of female adult mites, the female adult mites were selected to continue feeding, and the male adult mites were selected to mate, the fecundity and mortality were observed every 24 hours until all the female adult mites died.

2.4. Preparation of samples for Enzyme assay

The female adult mites (as described in Sect. 2.3) were treated with bifenazate at concentrations of LC_{10}, LC_{30} for 24 hours, and the survivors were picked up. 150 individuals were placed in a 1.5ml centrifuge tube. The collected samples were treated with liquid nitrogen and stored at − 80°C. There were four treatments in the experiment, and three replicates were set for each treatment.

2.5. Measurement of Tissue Total Protein

The purchased total protein extraction kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the total protein content of the sample. PBS buffer (0.05 mol/L, pH = 7) was added into the centrifuge tube containing 150 mites. After full homogenization, the homogenate was centrifuged at 4 ℃ and 14000 rpm for 15 min, and then the supernatant was taken as the enzyme solution to be tested. The sample was added according to the sample adding system in the kit instructions. The reading was performed at 562 nm with Perkin Elmer.

2.6. Measurement of antioxidant enzymes activities

2.6.1. Catalase(CAT)

According to the instruction of CAT test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China)., the activity of CAT was determined by ammonium molybdate method. Absorb 200 μL of reaction mixture and add it into 96 well plate. Read the OD value at 405 nm, repeat 3 times, and take the average value. The activity of CAT was calculated.

2.6.2. Superoxide dismutase (SOD)

Refer to the instruction of SOD activity test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Mix the solution well, place it at room temperature for 10 min, absorb 200 μL accurately, and add the reaction solution into 96 well plate. At the wavelength of 550 nm, read the OD value of absorbance, repeat for 3 times, and take the mean value. The activity of SOD was calculated.

2.6.3. Peroxidase (POD)

The activity of POD was determined according to the instruction of POD assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). After the solution was mixed, centrifuged at 3500 rpm for 10 min, 200 μL supernatant was added into 96 well plate. Read the OD value at the wavelength of 420 nm, repeat three times, and take the mean value. The activity of pod was calculated.

2.7. Measurement of detoxifying enzyme activities

2.7.1. Carboxyl esterase (CarE)

According to the instructions of CarE assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the activity of CarE was determined by spectrophotometry. Fully mix the solution, take 5 μL supernatant and 1000 μL preheating working solution into 1ml glass cuvette in turn, quickly mix them, and read the absorbance value after 10 s and 190 s at 450 nm. The change of absorbance value is the measured value, repeat for 3 times, and take the average value. Calculate the vitality of CarE.

2.7.2. Glutathione S-transferase (GSH-ST)

The activity of GSH-ST was determined according to the instruction of GSH-ST assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Mix the solution well, place it at room temperature for 15 min, absorb 200 μL reaction solution and add it into 96 well plate. Reading the OD value of absorbance at 412 nm, repeat 3 times, and take the average value. The activity of GSH-ST was calculated.

2.7.3. Cytochrome P450 (CYP450)

According to the instructions of insect cytochrome P450 Elisa kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the content of cytochrome P450 was detected by competitive method. Read the OD value of absorbance at the wavelngth of 450 nm, repeat for 3 times, and take the average value. The determination should be carried out within 10 minutes after the termination of the solution. According to the concentration and OD value, the standard curve equation was calculated to calculate the activity of CYP450.

2.8. Date analyses

SPSS software (version 20.0) was used to analyze the probability of the values obtained from the bioassay, ANOVA was used to calculate the statistically significant mean value, and Student Newman Keuls (SNK) test was used to determine the significant difference between the treatments. The original data in all individual life tables were analyzed according to the theory of gender life table in specific age stage.

3. Results

3.1. Toxicity of bifenazate to the third instar female adults of P. citri

The toxicity of bifenazate to the third instar female adults of *P. citri* was stronger (Table 1). The median lethal concentration (LC_{50}) was 11.915 mg/L. the concentrations leading to 10% and 30% mortality were 3.625 mg/L and 7.015 mg/L, respectively.

3.2. Sublethal concentration of bifenazate effects on the life-history traits of P. citri

Through bioassay of the third instar female adults of *P.citri*, we obtained low and median lethal concentrations. Table 2 and table 3 show the effects on the life table parameters of F_1 offspring of *P.citri*. The results showed that compared with the control group, there was no significant difference in the average immature development duration of female adults of *P.citri* treated with LC_{10} and LC_{30} (Table 2) Compared with adult pre-oviposition (APOP) and total pre-oviposition (TPOP) of control group, pre-

oviposition and total pre-oviposition of LC_{10} and LC_{30} treatment were significantly prolonged. However, compared with the control, the maturity (13.011 d), longevity (23.194 d) and fecundity (5.178 eggs/female) were higher, LC_{10} and LC_{30} treatment significantly reduced maturity, longevity and fecundity (9.011 d, 19.706 d, 4.309 eggs/female and 7.744 d, 18.789 d, 3.413 eggs/female).

Compared with the control, the net reproductive rate (R_0) and average generation time (T) of female adult mites treated with LC_{10} and LC_{30} were significantly decreased, while the intrinsic rate of increase (r) of insects treated with LC_{10} had no significant difference; however, The intrinsic rate of increase (R), limited rate of increase (λ) for the LC_{30} treated group significantly decreased for the control group (Table 3); the population doubling time (DT) of LC_{30} group were prolonged, but there was no significant difference between LC_{10} group and control group.

As shown in Fig. 2, the probability of newborn eggs surviving to x age and developing to j stage is expressed by the survival rate of specific age stage (s_{xj}), and the survival curves of different age stages overlap obviously; Compared with the control group, the survival rate of female adult mites in LC_{10} and LC_{30} treatment groups is relatively low.

Age-specific survival rate (l_x), age-specific fecundity (m_x) and age-specific fecundity (l_xm_x) can be reflected in Fig. 3. l_x is the survival rate of newborn eggs to the age of x, m_x is the total fecundity of the population to the age of x, l_xm_x is the result of the multiplication of l_x and m_x; l_x and m_x of LC_{10} and LC_{30} treatment are lower than the control, l_x and m_x show a downward trend with the increase of concentration.

3.3. Sublethal concentration of bifenazate effects on protective enzyme activities of *P. citri*.

The CAT□SOD □POD activities of the third instar female adults *P.citri* treated with bifenazate was determined after 24 hours. As shown in Fig. 4, the CAT activity of LC_{10} and LC_{30} treatment increased significantly compared with the control; the SOD activity of LC_{10} treatment increased, but the SOD activity of LC_{30} treatment group had no significant differences. Moreover, there was no significant difference in POD activity in the LC_{10} treated group compared with the control, However, POD activity was significantly higher in the LC_{30} treated group.

3.4. Sublethal concentration of bifenazate effects on detoxifying enzyme activities of *P. citri.*

The activities of CarE, GSH-ST and CYP450 of the third instar female adults *P.citri* treated with bifenazate were determined 24 hours later. As shown in Fig. 5, the activity of CarE in all treatment groups increased significantly compared with the control; the activity of GSH-ST in LC_{10} treatment increased, while in LC_{30} treatment decreased significantly; the activity of CYP450 in all treatment decreased compared with the control.

4. Discussion

In pest control, it is important to assess the sublethal effects of insecticides on pest populations, and life table analysis is an important technique for effective assessment [13–15]. Fecundity, intrinsic growth rate (r), finite rate of increase (λ), and net reproductive rate (R_0) are several key parameters for evaluating population growth, development, and reproduction [14, 16].

Our toxicity assay results showed that bifenazate is a potential insecticide for effective control of *P. citri*; However, insecticide has not only acute toxicity, but also the sublethal effects[8, 17, 18]. In the current study, the fecundity of *P. citri* was decreased in the LC_{10} or LC_{30} treated groups, and the same response was observed in pests treated with other insecticides.[19–22] For example, the low lethal concentrations (LC_{20}□LC_{30}) of chlorfenapyr inhibit *T. urticae* development and reproduction[23], A low lethal concentration (LC_{30}) of cyantraniliprole significantly inhibited fecundity in *Helicoverpa assulta*[10]; In contrast, many studies have shown that low concentrations of insecticides promote pest fecundity, for example, treatment with LC_{10} and LC_{20} of spinetoram reduces the time of development of spotted mites from eggs to adults and promotes their fecundity [11]. *B.odoriphaga* reproduction was stimulated by chlorfenapyr[24], In this study, we found that the sublethal concentration of bifenazate can effectively inhibit the increasing population of *P. citri*. This result showed that the logical application of insecticides is integral to *P. citri* management.

Insecticide stress has an impact on insect development, fecundity and population parameters. In addition, they can also affect the activities of antioxidant enzymes and detoxification enzymes[25]. *P. citri* exposure to different sublethal concentrations of bifenazate (LC_{10}, LC_{30}) resulted in a significant increase in the activity of antioxidant enzymes, which was reduced near death (Fig. 4). Chemical stress causes the body to start its own defense system, produce a lot of reactive oxygen species, increase SOD activity and produce H_2O_2, which requires CAT and POD decomposition to achieve the relative balance of the body; when the concentration of chemical continues to rise, the self-defense ability will be weakened, resulting in the inhibition of protective enzyme activity[26, 27]; This is consistent with the previous studies on other pests using other insecticides[28].

At the same time, it can also affect the activity of detoxification enzymes; CYP450, GSH-ST, and CarE are three key supergene families in insect detoxification metabolism[29, 30]. GSH-ST is a family of enzymes that can catalyze the binding reaction of reduced glutathione (GSH) compounds, which has electrophilic properties and can catalyze the hydrolysis of esters, sulfates and amides. GSH-ST and CarE are the main enzymes involved in pesticide metabolism[31, 32]. Previous studies showed that the activities of CarE and GSH-ST were up-regulated in *Sogatella furcifera* treated with buprofezin at sublethal concentrations (LC_{10} and LC_{25}) for 48 h, which increased with insecticide concentration rising[33]. In the present study, the activities of CarE and GSH-ST increased significantly after exposure to different sublethal concentrations (LC_{10}, LC_{30}) of bifenazate, and decreased when approaching death (Fig. 5). In addition, CYP450 is a multifunctional enzyme, which plays an important role in the formation of insecticide resistance[34]. In a variety of animal cells, it has been proved that a variety of insecticides can induce the production of reactive oxygen species (ROS), and then induce oxidative stress[35]. ROS attack, protein

and lipid lead to oxidative damage, which destroys the integrity of enzyme structure and reduces enzyme activity.[36] At present, the results showed that compared with the control group, the CYP450 enzyme activity in the treatment group decreased significantly with the increase of the concentration of bifenazate. For example, when locusts were exposed to different sublethal doses of chlorpyrifos, the activity of CYP450 enzyme decreased with insecticide concentration increasing [37]. Therefore, these results provide a basis for further study on the molecular characteristics of antioxidant enzymes and detoxification enzymes of *P. citri*, and it is of great significance to understand the relationship between insecticides, antioxidant enzymes and detoxification enzymes.

In summary, bifenazate not only showed acute toxicity to *P. citri*, but also had sublethal effect. This study showed that sublethal concentration of bifenazate could significantly affect the development duration and fecundity of *P. citri*. In addition, the activity of CYP450 in *P. citri* exposed to sublethal concentration was inhibited. Therefore, it is necessary to study bifenazate effects on CYP450 and reproductive related genes in *P. citri* at mRNA level in the future.It is an effective way to control the growth and development of *P. citri*.

Declarations

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Author contributions

Hongyan Wang, Tianrong Xin, Zhiwen Zou, Jing Wang and Ling Zhong. conceived and coordinated the study, evaluated the patients, and wrote the paper. Hongyan Wang performed the experiments. Bin Xia. revised the paper. All authors reviewed the results and approved the fnal version of the manuscript.

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Tables

Table1. Toxicity of bifenazate on female adults of *Panonychus citri*

Insecticides	Concentration mg/l(95%CL)-1			LC-P equation	$\chi 2$	R
	LC_{10} mg/l	LC_{30} mg/l	LC_{50} mg/l			
bifenazate	3.625 (0.887-5.325)	7.015 (3.683-9.514)	11.915 (8.590-16.348)	y=-2.453+2.279x	19.222	0.906

Table2. Life tables of *Panonychus citri* exposed to sublethal concentrations of bifenazate.

stage	control	LC_{10}	LC_{30}
	Mean ± SE	Mean ± SE	Mean ± SE
Egg duration (days)	(4.900 ±0.050) a	(4.978 ±0.052) a	(5.056 ±0.046) a
Larve duration (days)	(1.350 ±0.044) a	(1.428 ±0.047) a	(1.406 ±0.058) a
Nymph duration (days)	(2.439 ±0.047) a	(2.500 ±0.045) a	(2.517 ±0.042) a
Female adult duration (days)	(13.011±0.078) a	(9.011 ±0.070) b	(7.744 ±0.072) c
Longevity (days)	(23.194±0.085) a	(19.706±0.088) b	(18.789±0.109) c
APOP (days)	(1.490 ±0.040) c	(1.789 ±0.040) b	(2.067 ±0.042) a
TPOP (days)	(10.181±0.072) c	(10.694±0.084) b	(11.045±0.085) a
Fecundity (eggs/female adult/day)	(5.178 ±0.035) a	(4.309 ±0.026) b	(3.413 ±0.029) c

Table3. The Sublethal effects of bifenazate on *Panonychus citri* population parameters

population parameters	control	LC_{10}	LC_{30}
	Mean ± SE	Mean ± SE	Mean ± SE
Net reproductive rate(R_0) (d^{-1})	(55.848±1.980) a	(34.815±1.826) b	(21.288±0.691) c
Mean generation time(T) (d)	(15.278±0.183) a	(14.380±0.144) b	(13.733±0.090) c
Intrinsic rate of increase(r) (d^{-1})	(0.263 ±0.005) a	(0.247 ±0.006) a	(0.223 ±0.002) b
Finite rate of increase (λ) (d^{-1})	(1.301 ±0.006) a	(1.280 ±0.008) a	(1.249 ±0.003) b
Doubling time	(2.637 ±0.047) b	(2.810 ±0.070) b	(3.117 ±0.030) a

Figures

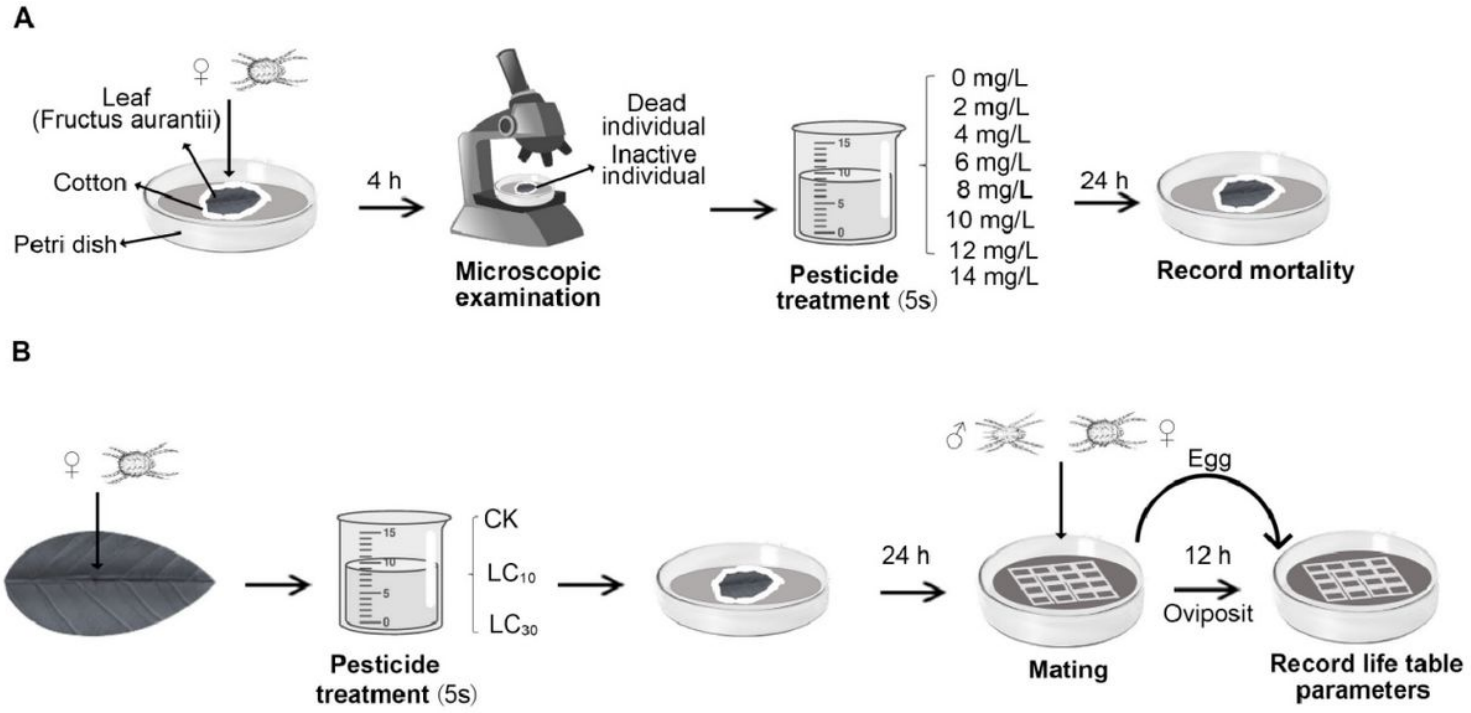

Figure 1

(A) Schematic diagram of bioassay method for bifenazate solution with seven concentrations (B) Life table observation of female adults of Panonychus citri in three treatments (CK, LC10, LC30).

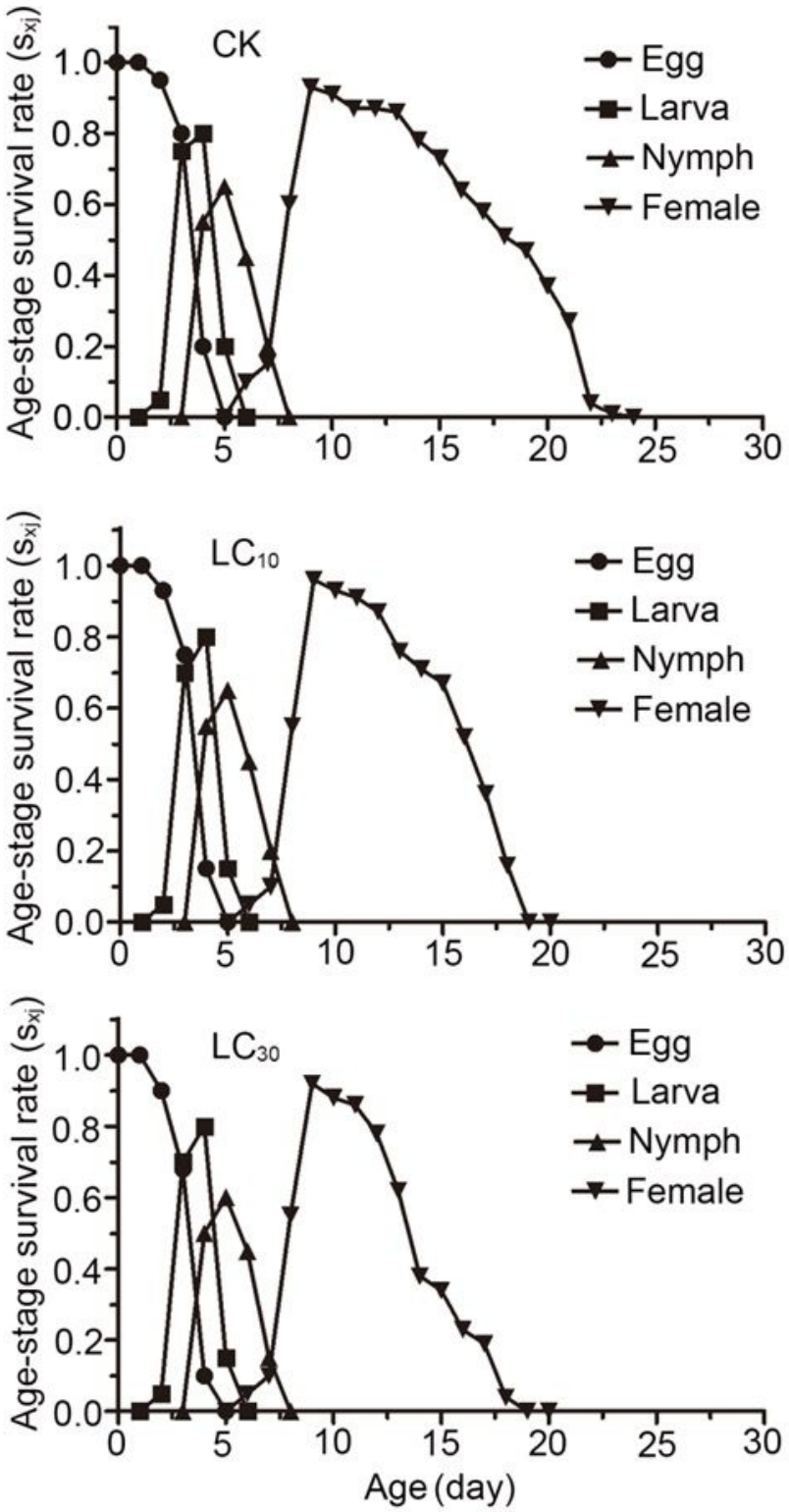

Figure 2

Age-stage specific s rate(sxj) of P. citri. after exposure to sublethal concentrations of bifenazate

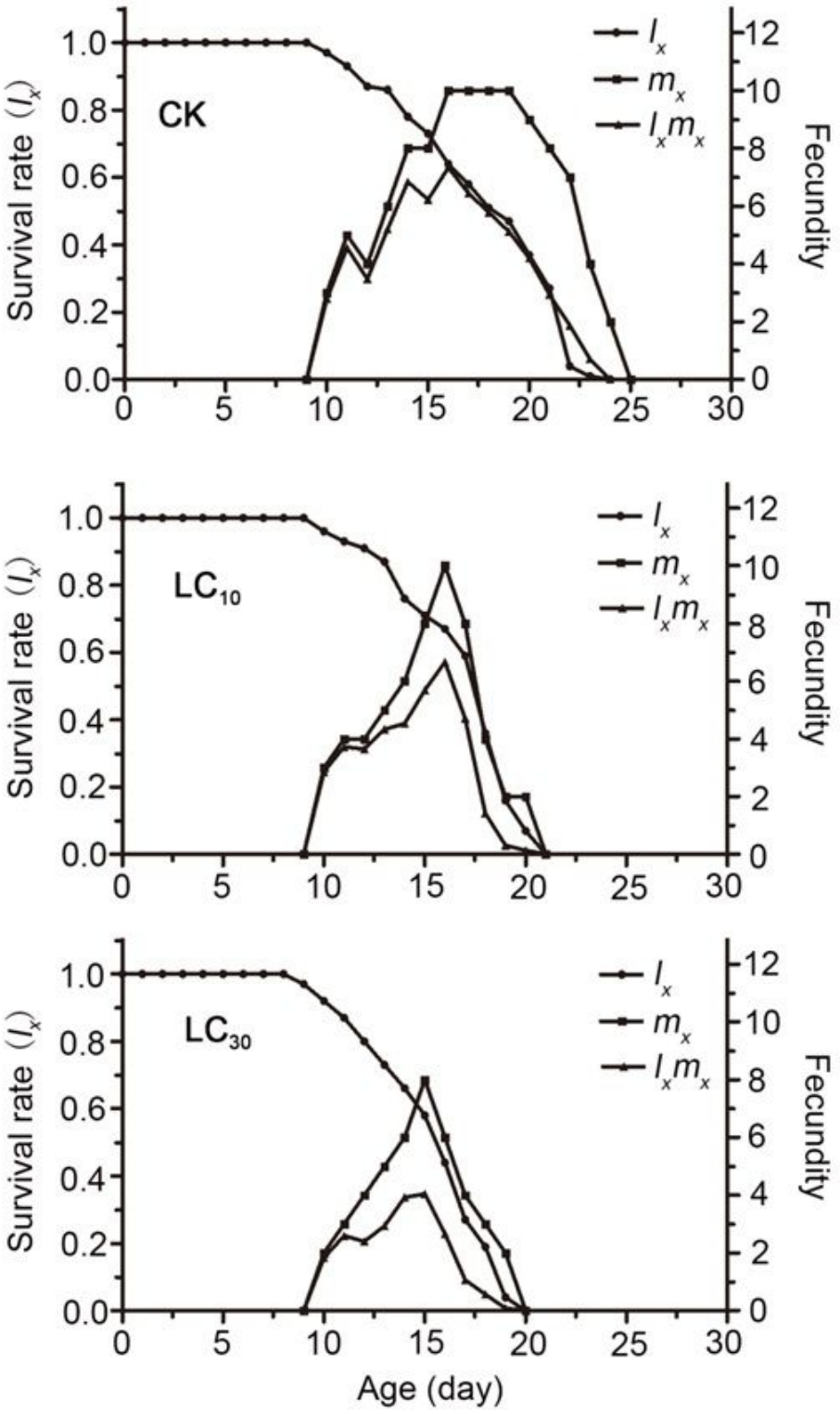

Figure 3

Age-specific survival rate(lx), age-specific feundity of the total population(mx), and age-specific maternity(lxmx) of P. citri. after exposure to sublethal concentrations of bifenazate.

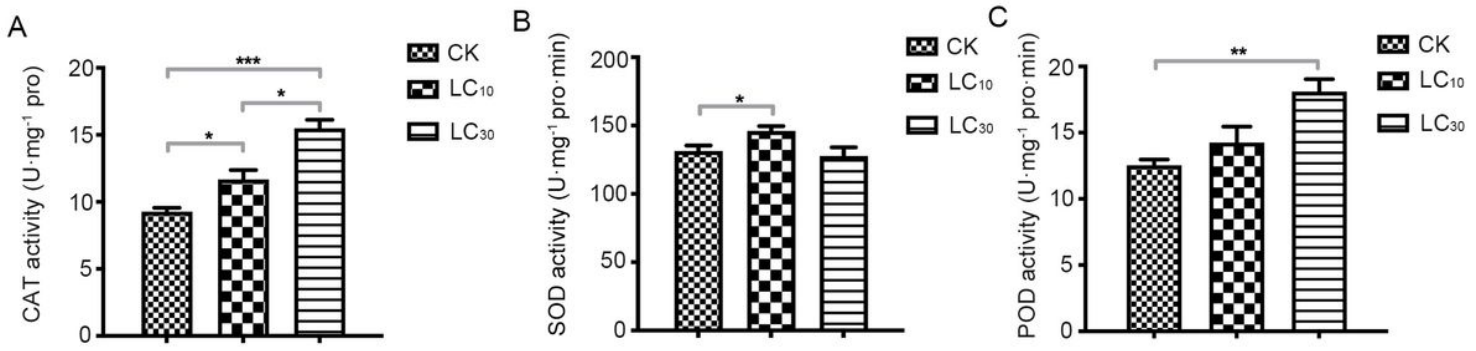

Figure 4

Effects of bifenazate stresses on antioxidant enzyme activity (U.mg-1 protein. min-1) of P. citri (CK served as control group, data are means±SE of three biological replications; Different letters above each bar indicate statistically significant difference by ANOVA followed by the Duncan's multiple range test) *P < 0.05, **P < 0.01, ***P < 0.001.(A:Catalase (CAT), B:Superoxide dismutase (SOD), C:Peroxidase (POD))

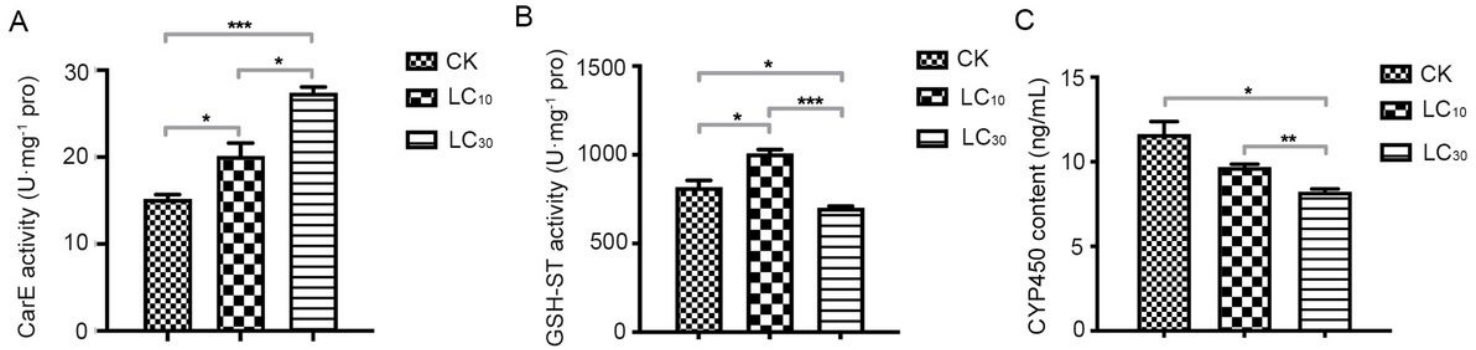

Figure 5

A-B□Effects of bifenazate stresses on carboxylesterase (CarE) □glutathioneS-transferase (GSH-ST) activity (nmol.mg-1 protein) of P. citri; C: Effects of bifenazate stresses on Cytochrome P450 (CYP450) content of P. citri. (CK served as control group, data are means±SE of three biological replications; Different letters above each bar indicate statistically significant difference by ANOVA followed by the Duncan's multiple range test) *P < 0.05, **P < 0.01, ***P < 0.001.