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Lethal and sublethal concentrations spirodiclofen stress may increase the adaptation of *Panonychus citri* (Acari: Tetranychidae)

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Research Paper

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Abstract

Panonychus citri is one of the most destructive pests in citrus orchards, exhibiting varying degrees of tolerance to numerous insecticides, such as spirodiclofen. To effectively manage pests, this study explores the response of *P. citri* to spirodiclofen stress from the perspectives of life history, enzymatic parameters, and reproduction. The effects of two concentrations (LC_{30} and LC_{50}) of spirodiclofen on the biological parameters of *P. citri* were evaluated by the life table method. The results showed that the development duration, fecundity, oviposition days, and lifespan were shortened, though the pre-oviposition period of two treatments was prolonged in comparison with the control. A significant decrease was recorded in the net reproductive rate (R_0) and the mean generation time (T) for the two treatments. Nevertheless, the intrinsic rate of increase (r) and the rate of increase (λ) were not significantly affected in the LC_{30} treatment, whereas they declined in the LC_{50} treatment. The enzyme activity assay resulted in higher activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and carboxylesterase (CarE), among the treatments than the control. In contrast, the treatments recorded lower cytochromeP450 (CYP450) and Glutathione S-transferase (GST) activities than the control. Furthermore, the study detected that relative mRNA expression of Vitellogenin (Vg) and Vitellogenin receptor (VgR) for two treatments were lower than the control. In summary, two concentrations of spirodiclofen inhibited progeny growth and fecundity of *P. citri*. Additionally, the results of this study may support further research on tolerance of *P. citri* in response to spirodiclofen stress.

Introduction

Panonychus citri (Acarina: Tetranychidae) is distributed worldwide and mainly feeds on the leaves of citrus (Pan *et al*., 2020). Presently, the key method for the control of *P. citri* is chemical control (Cheng *et al*., 2022). The enhanced application of pesticides prevents further damage, but it has several undesirable consequences, such as impacting natural enemies and non-target organisms. Meanwhile, the increased use of insecticides has led to widespread resistance of *P. citri* to insecticides (Quesada and Sadof, 2019). This emphasises the need to adopt effective acaricides for the control of *P. citri* (Andrade *et al*., 2018).

Spirodiclofen is an acaricide that belongs to the spirocyclic tetraelectron acid family and works by interfering with lipid biosynthesis (Amaral *et al*., 2020). Lipids constitute approximately 30–40% of the dry weight in insects (Ziegler and Antwerpen, 2006), with a higher proportion in mites. Consequently, this acaricide is widely used worldwide for controlling *P. citri* and mites in other crops, making it one of the most commonly used acaricides in China (Claudiane *et al*., 2021). However, its extensive use has also increased the frequency of misuse and overuse of insecticides. This improper usage has led to insects being exposed to lethal and sub-lethal concentrations of the insecticides, thereby becoming a key factor in accelerating pest resistance development. Previous research has shown that field populations of *P. citri* have relatively low resistance levels to spirodiclofen, but there is a possibility of developing higher levels of resistance in the future (Alavijeh *et al*., 2020). Field populations of phytophagous mites have also been confirmed to possess tolerance to spirodiclofen in regions such as the United States, Germany, Brazil, China, Turkey, and Iran (Cheng *et al*., 2022). Therefore, exposure of *P. citri* to lethal and sub-lethal concentrations of spirodiclofen in the field cannot be avoided. Assessing the potential lethal and sub-lethal effects of spirodiclofen on *P. citri* is crucial for a comprehensive analysis of the effectiveness of this insecticide.

Sublethal effects are defined as impacts (either physiological or behavioural) on survival individuals when exposed to a toxicant at low or sublethal concentration/dose (Desneux *et al*., 2007). The sublethal effects of pesticides on pests are not only reflected in the direct killing, but also affect their reproduction, lifespan, and physiological characteristics (Wang *et al.*, 2016; Dong *et al.*, 2017), as well as changes in their detoxification and antioxidant enzymes (Ma *et al.*, 2019). Hormesis, the stimulation of performance when an organism is exposed to low levels of agents, is regarded as the main mechanism for pest population resurgence (Cordeiro *et al.*, 2013). For example, some researchers have suggested that the sublethal concentrations of insecticides might incite insect outbreaks (Bartle, 1968). Currently, the lethal and sublethal effects of spirodiclofen on *P. citri* have not been determined.

Here, we used an age-stage, life table to evaluate the lethal and sublethal effects of the spirodiclofen on the life table parameters of *P. citri*, including development time, survival rate, longevity, fecundity, and hatchability. The results will help to provide a comprehensive assessment of this new insecticide for integrated pest management (IPM) and chemical applications in the field. Furthermore, understanding the activities of several key antioxidant enzymes and detoxification enzymes in *P. citri* can provide the basis for mitigating or reducing resistance to potent acaricides (Rasheed *et al.*, 2020).

Materials and methods

Mite and pesticide

In 2019, a stable population of *P. citri* was established by collecting a colony from Nanchang University of Nanchang, Jiangxi Province, China and maintaining them through continuous breeding. The population of *P. citri* were fed citrus tender leaves and were kept in an artificial climate box with the following environmental conditions: a temperature of 26 ± 1°C, a relative humidity (RH) of 70 ± 5%, and a photoperiod of 16:8 hours. The population was not exposed to any pesticides until the present study (in 2021), which was considered the acaricide-susceptible strain (SS).

The acaricide used in the bioassay was spirodiclofen (24%SC), which was purchased from Bayer Crop Science (China) Co. Ltd.

Bioassay

The modified leaf dipping method was used for the bioassay on the *P. citri* population (Yamamoto *et al.*, 1995). The adult females were transferred to citrus leaves ($n = 45$, three repetitions per treatment). Based on the pre-experiment, the acaricide was diluted to six concentrations (fig. 1). The control was performed on leaves with adult females soaked in triton X-100 solution. Immerse the leaves with *P. citri* in the prepared insecticide for 5 s (Wang *et al.*, 2021). The treated *citrus* leaves were placed on the prepared *Petri* dishes ($d = 15$ cm) in an air-conditioned room. After 24 h, the mortality of the *P. citri* population was recorded, and if their legs did not move, they were considered dead. The mortality data were used for LC_{30} and LC_{50} determination. All treated-individuals are raised through fresh citrus leaves (not exposed to any insecticides).

Changes in life table parameters

The adult females ($n = 100$, three repetitions per treatment) were transferred to the leaf dish and soaked with two spirodiclofen concentrations (LC_{30} and LC_{50}) by the leaf dipping method refer to the method described 2.2 (fig. 1A), the control was performed on leaves with adult females soaked in triton X-100 solution. Following 24 h, survivors (newly emerged 3-day-old adult females) were selected to continue feeding on the fresh-rearing platform. Meanwhile, adult males (without insecticide treated) were selected for mating. Eggs produced were cultured independently 12 h after mating ($n = 30$). The adult females (with insecticide treatment) were mated with adult males as soon as they

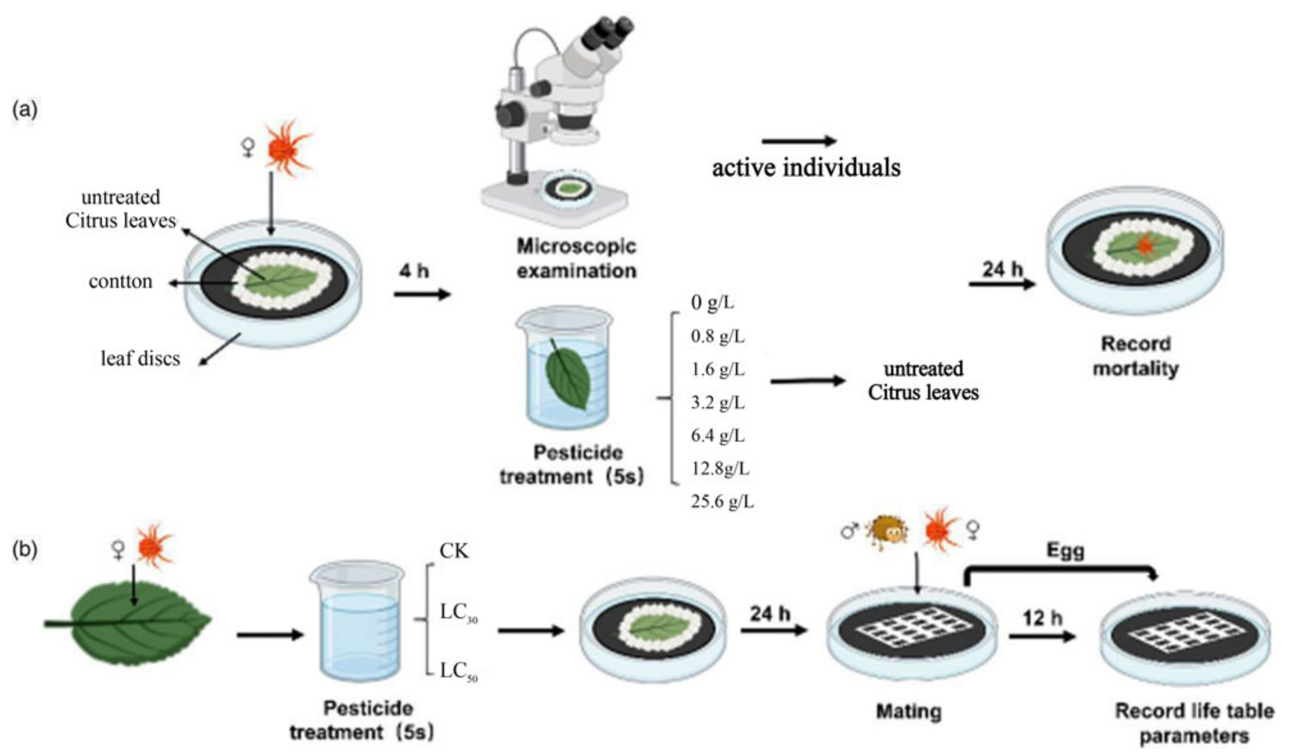

Figure 1. (A) Bioassay method schematic diagram (B) An observation of the life history of adult females of *Panonychus citri*.

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reached maturity, and they were observed every 24 h until died (fig. 1B).

Enzyme activity assay

3-day-old adult females were exposed to two concentrations of spirodiclofen (LC_{30} and LC_{50}) and control for 24 h, the prepared samples ($n = 300$, three biological repetitions per treatment) were frozen in liquid nitrogen and stored at −80°C in one 1.5 mL centrifuge tube to create the crude enzyme solution.

After calculation of protein concentration (BCA Protein Assay Kit- A045, Nanjing Jiancheng Bioengineering Institute, China), the supernatant of required volume was used to measure the enzyme activities of SOD, CAT, POD, CarE, GST, and CYP450 by commercial assay kits A001, A007, A084, A133, A004, and H303(Nanjing Jiancheng Bioengineering Institute, China). According to the instruction of CAT, SOD, POD test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), antioxidant enzymes activities: CAT, SOD, POD and detoxifying enzyme activities: CarE, GST, CYP450 were measured (Qi *et al.*, 2020). Using a photometer set at 450 nm, 550 nm, 420 nm, 450 nm, 412 nm, 450 nm, the amount of CAT, SOD, POD, CarE, GST, CYP450 in *P. citri* was determined. The change of absorbance value is the measured value, repeat for three times, and take the average value. Calculate the activity of six enzyme activities.

Gene expression analysis

The StepOnePlus real-time quantitative PCR machine was used to determine the mRNA level of some genes (such as Vg and VgR genes) after two concentrations of spirodiclofen exposure.

The 200 adult females were used to extract total RNA by Trizol reagent.(Shenggong Biological Technology Co. Ltd., Shanghai, China) following the manufacturer's specifications, and quantification was then performed using a NanoDrop 2000 spectrophotometer. In the following step, reverse transcription of RNA is performed using PrimeScript RT Reagent Kit with gDNA Eraser in a 20 µl reaction volume (TaKaRa, Shiga, Japan). Every RT-qPCR was carried out in a 20-µL mixture. The following were the qPCR cycling parameters: 95°C for 10 min, then 40 cycles of 95°C for 30 s and 60°C for 30 s. Primers were designed as shown in table 1, with the GAPDH and EF-1FA genes as internal reference genes. Relative quantification was calculated using the comparative $2^{-\triangle\triangle Ct}$ method (Zhao *et al.*, 2018b).

Statistical analysis

The life history parameters were counted, including age-stage specific rate (s_{xj}), Age-specific survival (l_x), age-specific reproduction (m_x), APOP (adult pre-ovipositional period), TPOP total pre-ovipositional period (from newborn egg to first oviposition). Population parameters including R_0, rm, λ, and T were also calculated as follows:

$$R_0 = \Sigma lxmx; \quad (1)$$

$$rm{:}\Sigma e - rm(x+1)lxmx = 1 \quad (2)$$

$$T{:}T = \ln R_0/rm; \quad (3)$$

$$\lambda{:}\lambda = erm. \quad (4)$$

The standard errors of raw data were calculated by using the bootstrap method with 100,000× resamplings, the paired bootstrap test was used to compare differences (Akkopru *et al.*, 2015). The TWOSEXMSChart program was used to analyse the raw data and population parameters (Chi and Liu, 1985).

Results

Toxicity of spirodiclofen in the adult females of Panonychus citri

Acute toxicity of spirodiclofen (24%SC) was observed in adult females *P. citri* (table 2). Concentrations resulting in 30 and 50% mortality were 3.898 g l^{-1} and 5.215 g l^{-1}.

Responses of spirodiclofen on population parameters of Panonychus citri

Through bioassay of *P. citri*, the low lethal concentration (LC_{30}) and median concentration (LC_{50}) were obtained. tables 3 and 4 present the lethal and sublethal effects on the F_1 generation of *P. citri*. The average immaturity time (The development stage of adult females before spawning) of *P. citri* treated with LC_{30} was not significantly different from the control (table 3). However, LC_{50} treatment was prolonged in egg duration. Compared to the control, LC_{30} and LC_{50} treatments significantly prolonged both oviposition period and total pre-ovipositional period. Compared with control in maturity (13.930 d), longevity (22.310 d), and fecundity (5.350 eggs/female), there were significant reductions in maturity (10.510 d and 5.620 d), longevity (21.430 d and 17.650 d), and fecundity (4.310 eggs/female and 2.680 eggs/female) after exposure two concentrations (LC_{30} and LC_{50}) of spirodiclofen.

Table 1. Primers for real-time quantitative PCR.

Genes	GenBank No.	Primer sequences(5' to 3')
PcVg	KC978893	Vg-F: GCCTCAAACGAAGCTCAATC Vg-R: AGCCAAAGCGTCGAGTAAAA
PcVgR	KC978894	VgR-F: TTGTTTCGATGGCTCAGATG VgR-R: TCACCGTGTGGACAATCAGT
GAPDH (Reference gene1)	HM582445	GAP-F: CTTFGGCCAAGGTCATCAAT GAP-R: CGGTAGCGGCAGGTATAATG
ELFIA (Reference gene2)	LOC100037748	ELFIA-F: GGCACITCGTCITCCACITC ELFIA-R: ATGATTCGTGGTGCATCTCA

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Table 2. Acute toxicity of spirodiclofen to adult females of *Panonychus citri*.

Acaricide	Concentration g/L (95% CL)		LC-P equation	χ^2	*R*
	LC_{30}	LC_{50}			
Spirodiclofen	3.898 (3.461–4.273)	5.215 (4.823–5.566)	$y = -0.5219 + 1.3878x$	17.43	0.94

The R_0 and *T* of adult females were significantly reduced on LC_{30} and LC_{50} treatments compared to the control. Adult females *P. citri* treated with LC_{30} showed no significant difference in rm and λ from the control, whereas they decreased in the LC_{50} treatment (table 4). It is clear that the survival rates at different ages are overlapping, as eggs are more likely to survive up to age x and develop to stage j when they occur at different age stages (fig. 2). Compared with the control, adult females survival rates in the LC_{30} and LC_{50} groups are relatively low. *Panonychus citri* survival rate (l_x), fecundity (m_x), and maternity rates ($l_x m_x$) after exposure to two concentrations of spirodiclofen are shown in fig. 3. The l_x and m_x of the treatment groups are lower than the control. Furthermore, both l_x and m_x decrease with the increase of concentration.

Responses of spirodiclofen on enzymatic activity of Panonychus citri

CAT, SOD, and POD activities were measured 24 h after spirodiclofen treatment. Difference in CAT between control and LC_{30} group was not significant (fig. 4). However, LC_{30} treatment significantly increased SOD activity compared with control, but both CAT and SOD activities of the LC_{50} treatment group showed a significant decline. The POD activity of the LC_{30} group has no significant difference which was compared with the control, whereas POD activity was significantly higher in the LC_{50} group. The activities of CarE, GST, and CYP450 of *P. citri* treated with spirodiclofen were determined. The activity of CarE in LC_{30} treatment increased compared with control, while it decreased significantly with increasing concentrations (fig. 5). Comparatively to the control group, all treatment groups showed a decrease in GST and CYP450 activities.

Vg and VgR relative expression

To determine how spirodiclofen affects the contents of Vg and VgR in *P. citri*, mRNA-relative expression of Vg and VgR were measured. Compared with CK, Vg and VgR expression were significantly decreased and showed a more significant decrease with increasing concentrations (fig. 6). In addition, significant concentration effects were also observed between the LC_{30} and LC_{50} treatments.

Discussion

The study of life table is a key aspect of insect population dynamics (Desneux *et al.*, 2007). The field of insect population dynamics is characterised by changes that are affected by many factors, including diet, temperature, light, and especially chemical pesticides (Mousavi *et al.*, 2020). Fecundity, *rm*, λ, and R_0 are several important parameters for assessing population dynamics (Papachristos and Milonas, 2008; Rahmani and Bandani, 2013).

The toxicity test indicated that fecundity and population parameters including rm, λ and R_0 of *P. citri* were decreased in the treated groups, and similar findings were observed in other insecticide-treated pests (Zhao *et al.*, 2018c). Chlorfenapyr, for example, inhibits *Tetranychus urticae Koch* growth and reproduction at low lethal concentrations (LC_{20} and LC_{30}) (Sani *et al.*, 2018). At low lethal concentrations (LC_{30}), cyantraniliprole significantly inhibits the fertility of *Helicoverpa assulta* (Dong *et al.*, 2017). In contrast, there have been several studies showing that low levels of pesticides can stimulate fertility. For instance, treatment with spinetoram LC_{10} and LC_{20} shortens the time for *T. urticae* to develop from egg to adult and increases their fecundity (Wang *et al.*, 2016). Chlorfenapyr stimulated *Bradysia odoriphaga* reproduction (Sani *et al.*, 2018). However, in the present study, the growth of *P. citri* can be effectively

Table 3. Spirodiclofen effects on *Panonychus citri* developmental time.

Parameter	Treatment		
	Control	LC_{30}	LC_{50}
Egg duration(d)	5.30 ± 0.20 b	5.96 ± 0.19 ab	6.58 ± 0.06 a
Larvae duration(d)	1.48 ± 0.15 a	1.41 ± 0.12 a	1.74 ± 0.04 a
Nymph duration(d)	2.49 ± 0.15 a	2.44= ± 0.11 a	2.78 ± 0.06 a
Immaturity period (d)	13.93 ± 0.23 a	10.51 ± 0.24 b	5.62 ± 0.06 c
Longevity(d)	22.31 ± 0.13 a	21.43 ± 0.41 b	17.65 ± 0.09 c
APOP(d)	1.61 ± 0.12 b	1.81 ± 0.08 ab	2.22 ± 0.04 a
TPOP(d)	10.18 ± 0.16 c	11.92 ± 0.22 b	13.25 ± 0.09 a
Oviposition period(d)	12.50 ± 0.37 a	9.47 ± 0.29 b	6.62 ± 0.06 c
Fecundity(eggs/female/d)	5.35 ± 0.25 a	4.31 ± 0.16 b	2.68 ± 0.03 c

The bootstrap paired test does not detect significant differences between means in a row following the same letter ($P > 0.05$). APOP, adult pre-ovipositional period; TPOP, total pre-ovipositional period (from newborn egg to first oviposition).

Table 4. Effects of spirodiclofen on population parameters of *Panonychus citri* offspring.

	Treatment		
Population parameters	Control	LC_{30}	LC_{50}
Net reproductive rate(R_0) (d^{-1})	54.80 ± 3.12 a	35.28 ± 1.54 b	11.21 ± 0.04 c
Mean generation time(T) (d)	1623 ± 0.28 a	14.23 ± 0.18 b	13.74 ± 0.05 b
Intrinsic rate of increase(rm) (d^{-1})	0.35 ± 0.03 a	0.27 ± 0.02 a	0.18 ± 0.03 b
Finite rate of increase (λ) (d^{-1})	1.42 ± 0.05 a	1.19 ± 0.03 a	1.07 ± 0.01 b

By the bootstrap paired test ($P > 0.05$), means in a row followed by the same letter are not significantly different.

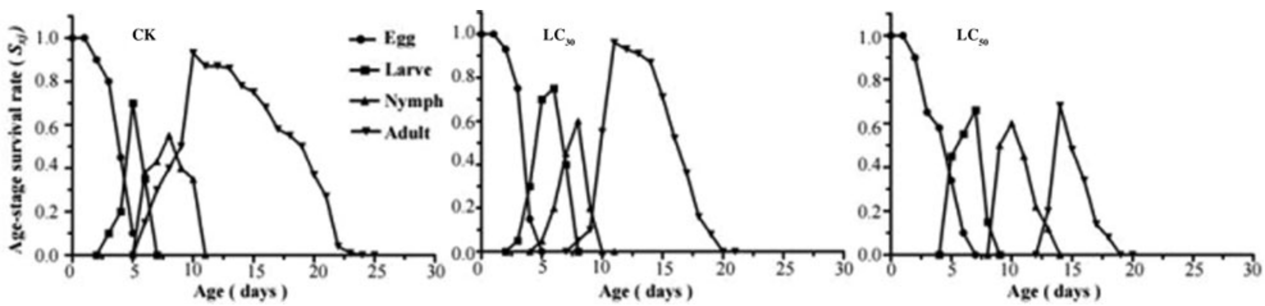

Figure 2. The age-stage specific rate (s_{xj}) of *Panonychus citri* at two concentrations of spirodiclofen.

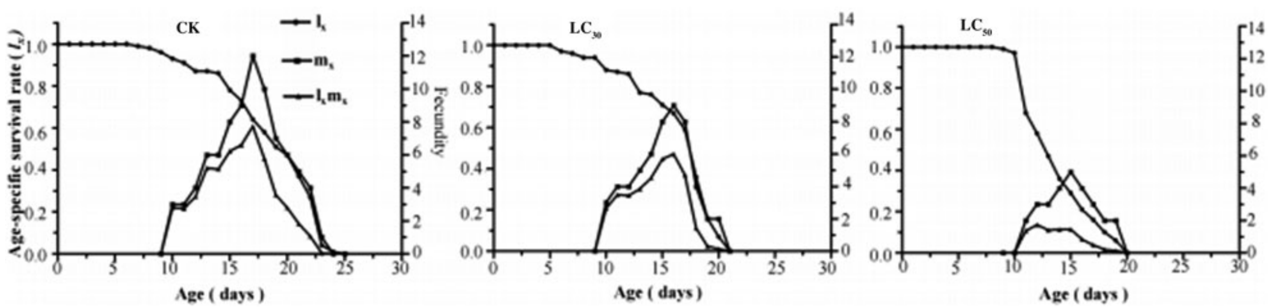

Figure 3. Age-specific survival (*lx*), age-specific reproduction (*mx*) and age-specific motherhood (*lxmx*) of *Panonychus citri* after exposure to two concentrations of spirodiclofen.

inhibited by two concentrations of spirodiclofen. As a consequence, different insects react differently to insecticide stress.

The fertility of the *P. citri* F1 generation decreased significantly. Adverse effects of drugs such as shorter lifespans and reduced fertility are often associated with their development (Zhang *et al.*, 2018). Adaptive costs of insecticide resistance, or the dominant disadvantage that accompanies the development of insecticide resistance, are associated with susceptible insects in a population. To gain more insight into this mechanism, our study measured the relative expression levels of two genes associated with growth and reproduction. Vitellogenin, a protein associated with reproduction, has traditionally been considered a suitable parameter for assessing the fertility of female insects (Zhao *et al.*, 2018a). For instance, the down-regulation of Vg levels adversely affected fertility in *Chilo suppressalis* and *Apolygus lucorum* (Huang *et al.*, 2016). The vitellogenin receptor is the main receptor of Vg function; notably, the down-regulation of VgR can inhibit Vg function. There was a significant reduction in mRNA expression of Vg and VgR in the experimental group compared to the control group. Combined with the significantly reduced fecundity of the treatments, these data suggest that the reduced expression of Vg mRNA in spirodiclofen-treatment may bear a significant effect on *P. citri* fecundity.

Various parameters related to insect population and development are affected by insecticide stress (Zhao *et al.*, 2018c; Zhang *et al.*, 2019, 2020; Ullah *et al.*, 2020). When insects are repeatedly exposed to the same pesticide, pesticide resistance will develop. Furthermore, the activities of the protective enzymes are influenced (Van *et al.*, 2006). Antioxidant enzymes are essential components of the insect immune system, preventing oxidative damage caused by foreign organisms. Toxin-induced reactive oxygen species (ROS) are removed by POD, SOD, and CAT antioxidant enzymes. As a result, the SOD-CAT-POD system acts as a first line of defence against ROS. H_2O_2 is produced when the body responds to chemical stress by generating ROS, upregulating the SOD activity, and activating defence systems of

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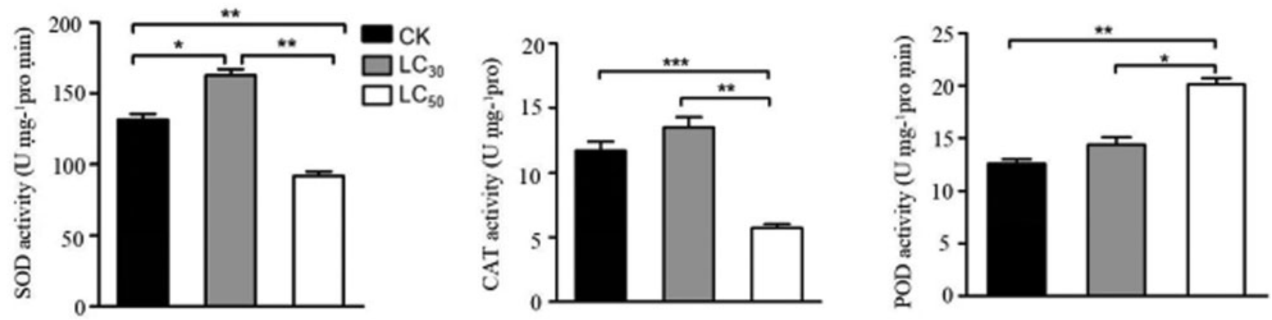

Figure 4. Response of protective enzyme activities of *Panonychus citri* to spirodiclofen stress (data are Means ± SE of three biological replications; different letters above each bar indicate statistically significant difference by ANOVA followed by the Duncan's multiple range test) $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

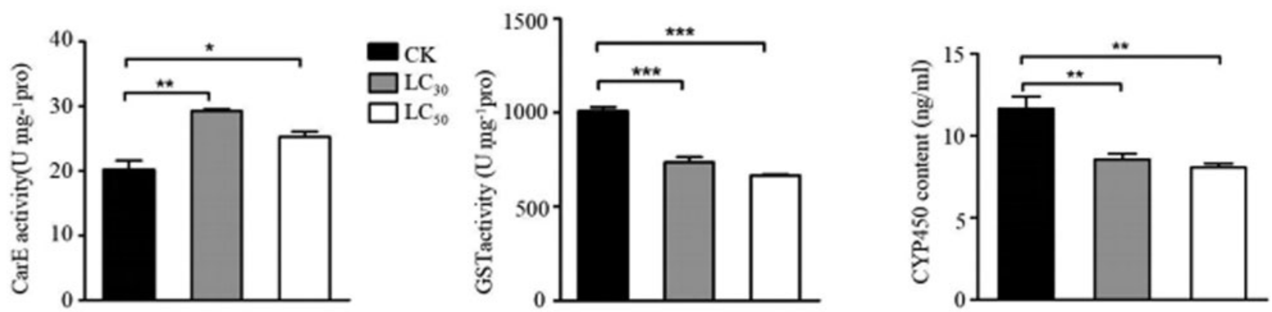

Figure 5. Response of detoxification enzyme activities of *Panonychus citri* to spirodiclofen stress. (data are Means ± SE of three biological replications; different letters above each bar indicate statistically significant difference by ANOVA followed by Duncan's multiple range test). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

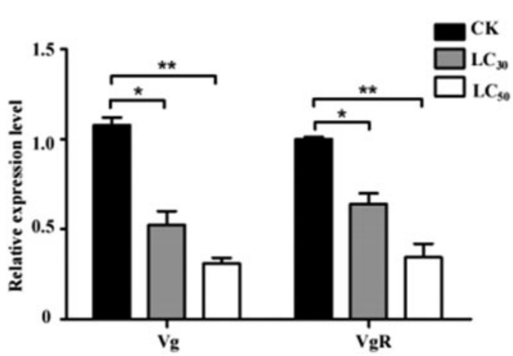

Figure 6. Relative expression level of Vg and VgR genes in adult females of *Panonychus citri* exposed to two concentrations of spirodiclofen. The bars represent the Means ± SE of three replications.

the body. A relative balance of the body needs to be regulated by the decomposition of CAT and POD. An increase in pesticide concentration weakened the self-defence mechanism and inhibited the function of the protective enzymes (Bolter and Chefurka, 1990). At low concentration, avermectin stimulates SOD activity. Conversely, at high concentrations SOD activity was inhibited. The same trend was observed for CAT activity. The study results showed that SOD and CAT have an inhibitory effect on LC_{50} treatment compared to lower concentrations (Ma *et al.*, 2014; Liu *et al.*, 2021).

Insecticide resistance is frequently caused by physiological changes that increase detoxifying enzymes like CarE, GST, and CYP450 (Zhao *et al.*, 2018c; Zhang *et al.*, 2020). In present study, when exposed to low lethal concentrations (LC_{30}) of spirodiclofen, CarE activity increased significantly, but decreased when near death. As concentrations increased, CYP450 and GST activities decreased (fig. 5). Toxins introduced into the body are dealt with by detoxifying enzymes in arthropods. CarE is phase II detoxification enzyme important in chemical metabolisation and detoxification (Papachristos and Milonas, 2008) The activities of CarE was upregulated after 48 h of treatment with low concentrations of buprofezin, and the activity decreased as insecticide concentration increased (Zhao *et al.*, 2018c). However, several hydrophobic toxins fail to be modified by CarE, causing lipid peroxidation in cell membranes. Therefore, the CYP450 and GST are important in the emergence of resistance genes (Ullah *et al.*, 2020). The production of ROS by insecticides induces oxidative stress in a wide variety of animal cells (Döker *et al.*, 2021). ROS attack causes oxidative damage to proteins and lipids, destroys structural integrity, and reduces enzyme activity (Goel *et al.*, 2005). For instance, in locusts exposed to different sublethal doses of chlorpyrifos, CYP450 enzyme activity decreased with increasing concentrations of the insecticide (Van *et al.*, 2006). Our study showed a significant decrease in the CYP450 enzyme activity with increasing spirodiclofen concentrations. The study assumed that GST and CYP450 activities in *P. citri* were a cause of tolerance against spirodiclofen.

In summary, spirodiclofen not only shows acute toxicity to *P. citri* but also exhibits sublethal effects. The present study showed that the population parameters of *P. citri* had adverse effects under two concentrations of spirodiclofen stress. Furthermore, reduced expression of Vg mRNA under spirodiclofen markedly affects *P. citri* reproduction. Thus, as a part of pest management programme, the responses of spirodiclofen stress show an effective way to control *P. citri*. However, the mechanism should be studied further in the future to clarify the effects against gene expression levels of detoxification enzymes and antioxidant enzymes.

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Competing interests. None.

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