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The Effect of Exogenous Melatonin in the Regulation of the Degradation of Residual Fungicide in Tomato

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Abstract: *Fungicides are widely used to control pathogen in modern agriculture. In particular, in the process of vegetable production, the use of fungicides could control a variety of diseases to increase crop yield. However, it is common that excessive and unsuitable application of pesticide cause serious pesticide residue in vegetables, which leads to problems of food safety and environment pollution. Therefore, it should not be ignored to reduce fungicide residue in vegetable. In order to confirm the effect of exogenous melatonin on degradation of residual fungicide in plant and explore the mechanism of regulation, Chlorothalonil was taken as experiment material in the present study, and exogenous melatonin was applied as pretreatment to investigate the mechanism of the degradation of residual Chlorothalonil in tomato. It is demonstrated that exogenous melatonin pretreatment could promote the degradation and metabolism of CHT residue in tomato plants by inducing the redox signal, improving the antioxidant system, enhancing antioxidant enzymes and increasing the ratio of GSH/GSSG to scavenge reactive oxygen species. And the activity of GST and GR enzymes were also enhanced to conduct detoxification, which result in a pronounced decrease in the residue of CHT in tomato.*

Keywords*: fungicide degradation, melatonin, redox, detoxification, antioxidant*

1. Introduction

Pesticides are widely used to control pathogen, pest and weed in modern agriculture, while the crop production may be lost by 80% without pesticides [1]. In the process of vegetable production, the use of pesticides could also control a variety of diseases and pests, and increase crop yield to a certain extent [2]. However, it is common that excessive and unsuitable application of pesticide cause serious pesticide residue in vegetables, which leads to a decline in the quality of agricultural products and by-products, and also results in pollution to the environment in the world [3]. Problems of food safety and environment pollution could endanger human health directly or indirectly [4] It is mentioned that pesticide residual in vegetable could cause both acute and chronic poisoning, which may cause cancer and other chronic diseases. Hence it should not be ignored that the problem of pesticide residues in vegetables rises a serious threat to food safety and human health. Therefore, how to reduce pesticide residue in vegetable has become an urgent problem.

Pesticide residues on the surface of vegetables and fruits could be removed by washing, soaking and peeling, while the other part of pesticides entering into fruits and vegetables cannot be removed by conventional methods [5]. However, low dose of toxic ingredients also do harm to human health directly or endanger human health indirectly, through the enrichment of the food chain in ecological environment, or even cause human congenital abnormalities and other problems [6]. Therefore, it has become an urgent problem to reduce pesticide residues in vegetables and explore the mechanism of degradation of residual pesticides in plants. Some studies focus on the degradation and mechanism of herbicide residue in plants [7]. In fact, it has been explored that plants have developed its detoxification mechanism to degrade and metabolic exogenous herbicide and relieve the negative impacts of herbicide [8].

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Glutathione plays a critical role in the regulation of herbicides metabolism, due to its function in both detoxification of toxic ingredients and protection of cells against free radicals [9]. However, most studies are concentrated in the degradation metabolism of herbicide residue, whose target is plant, while there are only a limited study focused on the degradation metabolism of pesticides in plants, especially on the degradation of fungicides. As a matter of fact, in the process of vegetable production, number types of germicidal pesticides are used to control plant diseases. Among them, Chlorothalonil (2,4,5, 6-tetrachloroisophthalonitrile, CHT) is widely used in the prevention of diseases in vegetable and other crop production, due to its efficient inhibition of pathogens growth. However, chemical properties of Chlorothalonil are stable, which is difficult to degrade under natural conditions because of low water solubility. As a result, it is also easy to form pesticide residue in vegetables. Therefore, the safety problem caused by chlorothalonil residue in vegetable cannot be ignored.

It is mentioned that hormone treatment could improve plant resistance to abiotic stresses and regulate the mechanism of exogenous substances. And Brassinosteroids has been demonstrated to be a positive regulation factor that promote the degradation of pesticides residue in vegetables in our previous study [1]. Although a great many studies indicate positive effects of exogenous melatonin on plant resistance to abiotic stresses, until now, little is known about the effect of exogenous melatonin in the regulation of the degradation of residual fungicide in vegetables [10-12]. And the regulation mechanism of exogenous melatonin in residual fungicide degradation in plants is still not clear. In order to confirm the effect of exogenous melatonin on degradation of residual fungicide in plant and explore the mechanism of regulation, Chlorothalonil was taken as experiment material in the present study, and exogenous melatonin was applied as treatment to investigate the mechanism of the degradation of residual Chlorothalonil in tomato.

2. Materials and methods

2.1. Plant materials

Tomato (Guanghui 101) seeds were grown in a mixture with peat and vermiculite (7:3). The tomato plants were cultured in the growth conditions: the temperature was kept at 25 °C with 120mol m/s light for 14 h in the daytime, and the temperature was kept at 20°C in the dark for 10 h.

2.2. Chemical treatments and sample harvesting

Tomato plants with 6 true leaves fully expanded were pretreated with melatonin (3 mM) as treatment (Sigma, USA), while deionized water was utilized as control to explore the effect of melatonin (MT) in the regulation of fungicide degradation. 24 h after pretreatment, tomato leaves were sprayed with chlorothalonil (CHT) at 11.2 mM with 30mL per plant (commercial CHT 75% active ingredient, Shenzhen Noposion Agrochemicals Co., Ltd.). The plant tissues were sampled at 0, 3, 6, 12, 24, 36, 48, 96 h (4d), and 168 h (7 d) after CHT treatment for biochemical analysis. And plant samples were harvested 7d after CHT application to analyze the residue of CHT in tomato plants.

2.3. Measurement of the content of GSH and GSSG

The content of GSH and GSSG were measured according to the method of Rahman and Kode (Rahman et al., 2007).

2.4. Assay of GST and GR activity

0.3 g leaf samples were extracted in 2 mL 50 mM PBS buffer (*p*H 7.5) with 10mM KCl, 1 mM EDTA, 5mM DTT, 0.5mM AEBSF and 1:4 insoluble PVPP to determine the activity of glutathione reductase (GR) and glutathione S-transferase (GST). The homogenates of plant tissue were centrifuged at 14000 rpm for 20 min, and the supernatants were used to analyze the activity of enzyme. The activity of GST was measured at the absorbance of 412 nm as described by Xia et al [13]. The activity of GR was assayed dependent on the rate of decrease at the absorbance of 340 nm according to Xia et al. [13].

2.5. Analysis of Antioxidant enzymes activity

0.3g leaf samples were extracted in 2 mL of 50 mM PBS buffer (*p*H 7.5), and then centrifuged at 12000 g for 20 min, and the supernatants were used to analyze the activity of enzymes. The activity of SOD, POD, CAT, APX, and MDAR were assayed according to the methods of Erkan et al. [14].

2.6. Investigation of the content of H_2O_2 and O_2

The level of H_2O_2 and O_2 were determined according to the methods of Zhu et al. [15].

2.7. Quantification of CHT in plant tissue

To assay the level of CHT residue in tomato, 10g tomato leaves were extracted with 80mL petroleum ether including 40 g Na_2SO_4 for 12 h and then filtered, and the filtrates were collected and dried with rotary evaporators. To analyze the level of CHT, N-hexane was used to dissolve pesticide, and the volume was adjusted to 5 mL, and gas chromatography (GC) with ECD and a capillary column (30m length, 0.32mm internal diameter and 0.25 μm film thickness) (Agilent, Santa Clara, CA, USA) was applied. Nitrogen (3.3 $mLmin^{-1}$) was employed as carrier gas, and the injector port temperature was set at 250 ℃, while the detector temperature was set at 300 ℃, and column temperature was raised from 80 to 260 ℃ (25 ℃/min), and then maintained for 3.8 min. CHT (Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China) was applied was used as standard. The level of residual CHT in plant tissue was determined according to the standard method [16].

2.8. Statistical analysis

Assay was all performed with three replicates. SPSS was used to analyze the statistic. The data were subjected to analysis of variance, and the means were compared using Tukey's test at the 5% level.

3. Results and discussions

3.1. Effect of melatonin pretreatment on chlorothalonil residue in tomato

Chlorothalonil is one of the most common fungicides, which could effectively act on common diseases in vegetable production, such as downy mildew and powdery mildew. In order to explore whether melatonin pretreatment do have positive influence in the fungicide residue degradation metabolism in plants, the content of CHT residue in tomato with or without melatonin were determined. Compared with the control, pretreatment with melatonin (MT) significantly decreased the residue of CHT in the tomato plants and the level of CHT residue reduced by 30.27% (Figure 1). The results revealed that exogenous MT could promote the degradation and metabolism of chlorothalonil effectively in tomato leaves and reduce the residue of chlorothalonil in tomatoes.

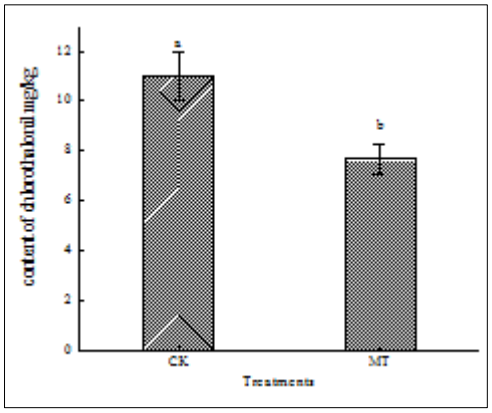

Figure 1. Effect of melatonin pretreatment on chlorothalonil residue in Tomato
Different letters indicate significant difference between the data (P< 0.05)

3.2. Effects of melatonin pretreatment on ROS production rate and the level of H_2O_2 in tomato leaves with CHT application

As shown in Figure 2, the production of ROS and the level of H_2O_2 in tomato leaves were induced with melatonin pretreatment, however, the level of which were significantly lower than control pretreated with water since 12-24 h after CHT application. Compared to the control, the ROS production rate of tomato leaves pretreated by exogenous MT was significantly increased during 0-3 h after CHT treatment, while the ROS production rate of tomato leaves pretreated with melatonin reduced since 6 h after CHT treatment, and the difference was significant between MT pretreatment and the control all the time except for 36 h and 4d. The level of H_2O_2 in tomato leaves pretreated with exogenous melatonin was induced between 0 and 12 h after CHT application, and the content of H_2O_2 was significantly different from that of the control all the time except for 3h. However, the content of H_2O_2 in tomato leaves pretreated with exogenous MT was significantly lower than that of the control since 24 h after CHT treatment. Therefore, it could be observed that MT pretreatment could induce short burst of ROS in plant, and increased the O_2^- production rate and H_2O_2 level in tomato leaves, while decreased significantly since 12 h after CHT application, when reactive oxygen species were eliminated gradually to prevent damage from fungicide stress.

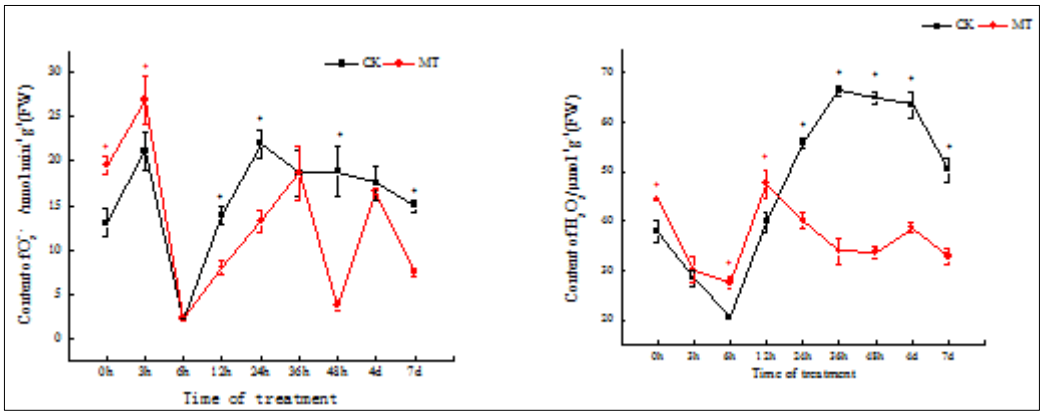

Figure 2. Effects of MT pretreatment on ROS production rate (a) and H_2O_2 level(b) in tomato leaves with CHT application

3.3. Effects of melatonin pretreatment on the rate of GSH/GSSG in tomato leaves with CHT application

In order to investigate whether glutathione is involved in the regulation of MT pretreatment in the degradation of CHT residue in tomato, GSH/GSSG ratio of CHT exposed plants were detected with MT pretreatment. As shown in Figure 3, compared with the control, MT pretreatment could significantly improve the GSH/GSSG ratio. GSH/GSSG ratio of tomato leaves was significantly higher than that of the control, and the distinct was significant all the time except for 4d after CHT exposure. In addition, the GSH/GSSG ratio of CHT exposed tomato plants pretreated with exogenous MT reached its highest peak (13.3) at 3 h after CHT treatment, which was as twice as that of the control. However, the GSH/GSSG ratio decreased slowly and stabilized at the subsequent time point. Thus, it could be inferred that exogenous melatonin could promote the degradation and metabolism of CHT residue in tomato plants by regulating the increase of GSH/GSSG ratio.

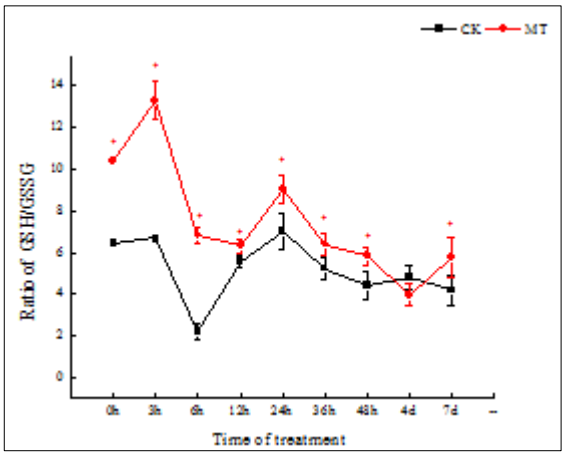

Figure 3. Effects of MT pretreatment on GSH/GSSG ratio in tomato leaves with CHT application

3.4. Effects of melatonin pretreatment on antioxidant enzymes activity in tomato leaves with CHT application

In order to reveal whether exogenous melatonin plays an important role through antioxidant system in regulating the degradation of fungicide residue in tomato, effects of melatonin pretreatment on antioxidant enzymes activity of tomato plants were analyzed (Figure 4). Although it was shown that various changes coupled with the extension of exposure time of CHT due to different antioxidant enzymes, MT pretreatment increased the activity of antioxidant enzymes in CHT exposed plants to different extent. It could be concluded that MT pretreatment increased the activity of SOD in CHT exposed tomatoes all the time, and there was a significant difference between 6 and 36 h after CHT treatment (Figure 4a). During the period (6-36 h), the activity of POD enzyme of tomato exposed to CHT was also significantly enhanced by pretreatment with MT, while inhibited in the other time (Figure 4b). Exogenous MT pretreatment also significantly promoted the activity of both APX and MDAR enzyme in CHT exposed plants in the period of 0-3 h and the activity of APX enzyme in the period of 12-48 h, while the activity of MDAR enzyme at the time of 6-24 h was significantly lower than that of the control, with no significant difference at the later stage (Figures 4c and 4d). It is interesting that the SOD, APX, POD, and MDAR enzymes in tomatoes with MT pretreatment all suggested to be increased first and then decreased, and the peaks of different enzymes appeared in a certain extent. Therefore, MT pretreatment could remove reactive oxygen species by regulating antioxidant enzymes and participate in the regulation of CHT degradation.

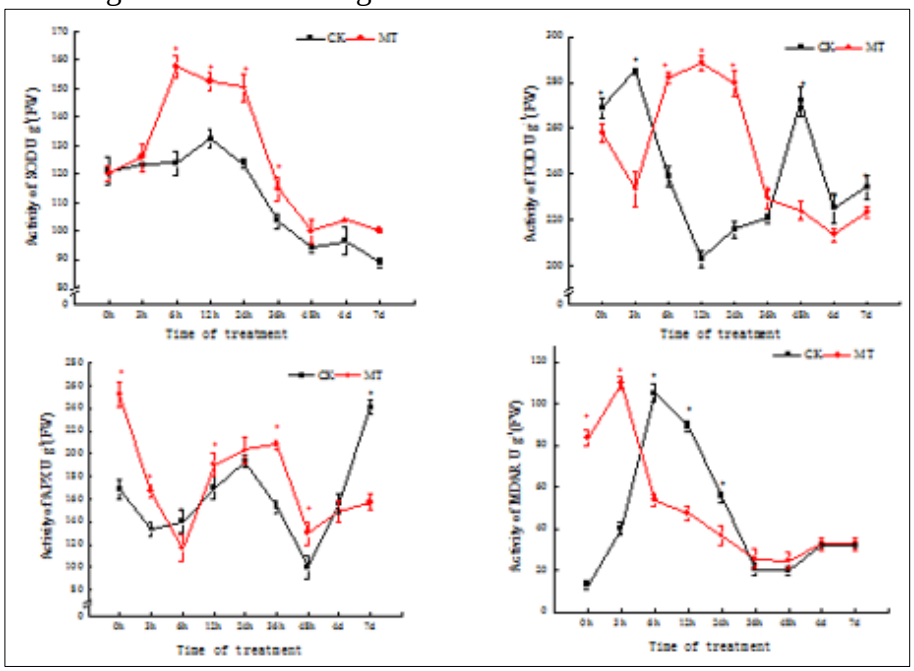

Figure 4. Effects of MT pretreatment on antioxidant enzymes activities of CHT exposed tomato

3.5. Effects of melatonin pretreatment on detoxification enzymes activity in tomato leaves with CHT application

As an important detoxification enzyme, Glutathione-S-transferase (GST) in plant could catalyze the reaction of the exogenous substance or its metabolites with the thiol group of reduced glutathione (GSH) in plant to prevent to be injured. At the same time, reduced glutathione (GSH) was oxidized into oxidized glutathione (GSSG), which could be reduced into GSH by glutathione reductase (GR), resulting in an increase in the content of GSH, as the substrate of GST enzyme, coupled with improvement of the antioxidant capacity of the plant. As shown in Figure 5, melatonin pretreatment increased the GST enzyme activity after exposed to CHT for 24 h. Although the activity of GST enzyme of tomato pretreated with MT was lower than the control during 3-12 h, which was significantly higher than that of the control since 24 h after CHT exposure. While the activity of GR enzyme in MT pretreated plants was significantly induced during the period of 24-48 h. Thus, exogenous melatonin could play a positive role in the degradation of residue CHT in tomatoes through regulating the activity of GST and GR enzymes.

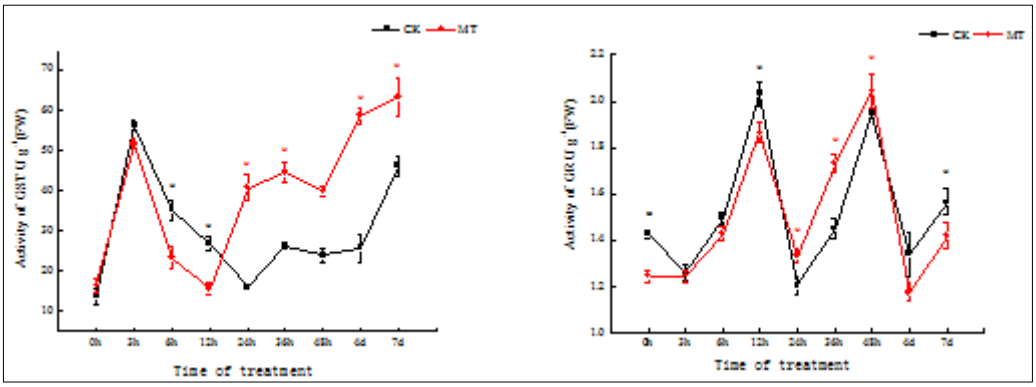

Figure 5. Effects of MT pretreatment on detoxification enzymes activity of CHT exposed tomato

3.6. Effects of melatonin pretreatment on Non-protein thiol content in tomato leaves with CHT application

The content of non-protein thiol in tomato could reflect the decomposition products of pesticide to a certain extent, which was associated with glutathione conjugates level. With the extension of CHT exposure time, the level of non-protein thiol in tomato leaves probably indicated an increased trend. Compared with the control, the content of non-protein thiol in tomato was significantly up-regulated with the pretreatment of MT after the application of CHT, particularly from 3 to 36 h and 4d after CHT treatment, the difference between treatments reached the significance level (Figure 6). All the results demonstrated that MT could increase the content of non-protein thiol, and enhanced the metabolism of CHT in tomato plant.

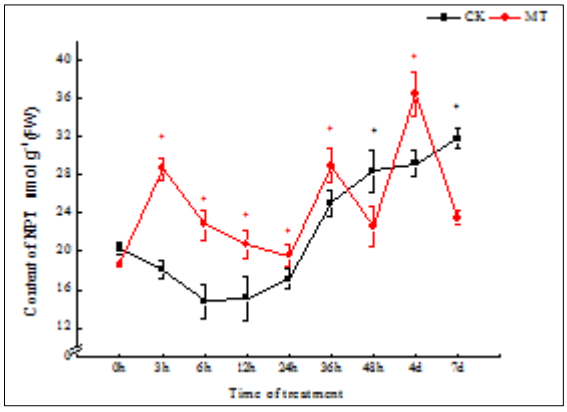

Figure 6. Effects of melatonin pretreatment on content of NPT in CHT exposed tomato

In fact, it has been explored that plants have developed detoxification mechanism to promote residue herbicide degradation and relieve the negative impacts [8]. However, until now there have been only limited reports on the regulation of degradation of fungicide residues in plant. Hormone has been explored to act as a positive regulation factor to improve plant resistance to abiotic stresses and regulate the mechanism of exogenous substances. It is mentioned that exogenous Brassinosteroids could effectively promote the degradation of pesticides residue in vegetables in our previous study [1]. Here the results also revealed that exogenous MT play an important and positive role in the degradation and metabolism of fungicides residue in plant, as a result of that the residue of chlorothalonil reduced effectively in tomato leaves of pretreatment with MT, a safe, pollution-free and edible substance.

Melatonin has been found to play an important role in plant growth regulation and stress resistance [17, 18]. It is indicated that MT could not only promote germination of *Phacelia tanacetifolia* seeds and adventitious root development of tomato [19, 20]，but also delay drought-induced leaf senescence in apple [21], enhance the resistance of water stress and promote lateral root formation in cucumber [22], alleviate cold stress in Arabidopsis [23], and protect red cabbage seedlings against toxic copper ion [24]. The result also demonstrated that exogenous MT could also alleviate fungicide stress and promote CHT detoxification and degradation in tomato leaves significantly. In addition, it could be observed that MT pretreatment induced short burst of ROS in plant with exposure to CHT, however, decreased the O_2 production rate and H_2O_2 level significantly in tomato leaves since 12 h exposed to CHT, resulted in the elimination of ROS to be less injured. It is well known that Melatonin (n-acetyl-5-methoxytryptamine) is small molecular substance with effective antioxidant, which could remove reactive oxygen species effectively, including hydrogen peroxide, peroxide anion, hydroxyl radical, single oxygen and other superoxide radicals [25, 26].

Additionally, it is mentioned that melatonin could accelerate the metabolism of superoxide free radicals by improving the activity of antioxidant enzymes in plants, to maintain the dynamic balance between the generation and disappearance of free radicals in plants and alleviate oxidative damage caused by abiotic stress [27]. In fact, MT pretreatment could remove reactive oxygen species by regulating antioxidant enzymes to participate in the regulation of CHT degradation. The result suggested that the activity of SOD, APX, POD and MDAR enzymes were effectively enhanced by MT pretreatment in tomato leaves with application with CHT in a certain extent. It is also reported that MT could increase the activity of antioxidant enzymes in cucumber exposed to salt stress, and promote seed germination under high salinity by regulating antioxidant systems to reduce the level of ROS and H_2O_2 in cucumber [28].

The content of NPT, associated with glutathione conjugates level, was significantly increased in MT pretreated plants. However, a pronounced decrease in the residue of CHT was observed. These phenomena provide an important evidence to confirm the participation of glutathione in CHT metabolism in tomato, and CHT is detoxified partly by the formation of glutathione conjugation. It has been mentioned that glutathione could react with a range of substrates including halides and other electrophilic compounds in the presence of the enzyme GST [7]. As a result, the reaction gives rise to the formation of soluble conjugates of pesticide molecule or its metabolites with natural plant constituents, which can be stored in vacuoles during Phase II reactions or conjugated to form non-extractable or bound residue in Phase III reaction to decrease water solubility of pesticide, thereby reduce their reactivity and toxicity [8].

Additionally, it is interesting that glutathione plays a double role in the CHT residue degradation in plant. It is well known that glutathione plays an important role in the regulation of redox homeostasis and redox sensing, although the mechanism in plant is not very clear [29]. It could be inferred that exogenous melatonin could promote the degradation and metabolism of CHT residue in tomato plants by regulating the increase of GSH/GSSG ratio and enhancing the activity of GST and GR enzymes since 12 h exposed to CHT. Moreover, GSH can be used as the substrate for GST-catalyzed reactions. As a result, the activity of GST enzyme may be influenced by GSH level and the rate of GSSG. While oxidized glutathione (GSSG) can be restored to reduced glutathione GSH (GSH), which also reacts to

the ratio of GSH/GSSG. It is also possible that the activity of GST and GR are modified by the redox status since they are also subjected to redox regulation [30]. However, glutathione status is involved in H_2O_2-triggered signal transduction, which would influence H_2O_2 concentration through its antioxidative function [31]. Accordingly, it is still ambiguous whether changes in glutathione status are themselves sensed or rather affect antioxidant enzymes through secondary effects on ROS availability [32-36].

4. Conclusions

In summary, the present data demonstrated that exogenous melatonin pretreatment could promote the degradation and metabolism of CHT residue in tomato plants by inducing the redox signal, improving the antioxidant system, enhancing antioxidant enzymes and increasing the ratio of GSH/GSSG to scavenge reactive oxygen species. And the activity of GST and GR enzymes were also enhanced to conduct detoxification, which result in a pronounced decrease in the residue of CHT in tomato.

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