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Exploration of Synergistic Pesticidal Activities, Control Effects and Toxicology Study of a Monoterpene Essential Oil with Two Natural Alkaloids

Jianwei Xu [1], Min Lv [1,*], Shanshan Fang [1], Yanyan Wang [1], Houpeng Wen [1], Shaoyong Zhang [2] and Hui Xu [1,*]

[1] College of Plant Protection, Northwest A&F University, Xianyang 712100, China
[2] Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, College of Life Science, Huzhou University, Huzhou 313000, China
* Correspondence: author: lvmin@nwsuaf.edu.cn (M.L.); orgxuhui@nwsuaf.edu.cn (H.X.)

Abstract: With the increasing development of pest resistances, it is not easy to achieve satisfactory control effects by using only one agrochemical. Additionally, although the alkaloid matrine (**MT**) isolated from *Sophora flavescens* is now utilized as a botanical pesticide in China, in fact, its pesticidal activities are much lower in magnitude than those of commercially agrochemicals. To improve its pesticidal activities, here, the joint pesticidal effects of **MT** with another alkaloid oxymatrine (**OMT**) (isolated from *S. flavescens*) and the monoterpene essential oil 1,8-cineole (**CN**) (isolated from the eucalyptus leaves) were investigated in the laboratory and greenhouse conditions. Moreover, their toxicological properties were also studied. Against *Plutella xylostella*, when the mass ratio of **MT** and **OMT** was 8/2, good larvicidal activity was obtained; against *Tetranychus urticae*, when the mass ratio of **MT** and **OMT** was 3/7, good acaricidal activity was obtained. Especially when **MT** and **OMT** were combined with **CN**, the significant synergistic effects were observed: against *P. xylostella*, the co-toxicity coefficient (CTC) of **MT**/**OMT** (8/2)/**CN** was 213; against *T. urticae*, the CTC of **MT**/**OMT** (3/7)/**CN** was 252. Moreover, the activity changes over time of two detoxification enzymes, carboxylesterase (CarE) and glutathione *S*-transferase (GST) of *P. xylostella* treated with **MT**/**OMT** (8/2)/**CN**, were observed. In addition, by scanning electron microscope (SEM), the toxicological study suggested that the acaricidal activity of **MT**/**OMT** (3/7)/**CN** may be related to the damage of the cuticle layer crest of *T. urticae*.

Keywords: matrine; 1,8-cineole; ternary complex; pesticidal activity; detoxification enzymes; SEM

Key Contribution: Joint pesticidal activities of **MT**–**OMT**–**CN** were related to the mass ratio of ternary complex and controlling pests. These results will lay the foundation for the future study of different combinations of **MT**–**OMT**–**CN** and the use of **CN** as a synergist with other bioactive plant natural products as pesticidal agents in crop protection.

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1. Introduction

Plutella xylostella Linnaeus (diamondback moth; Lepidoptera: Plutellidae) as the major migratory insect pest mainly endangered cruciferous vegetables and caused economic loss in the world of up to US$ 4–5 billion annually [1,2]. *P. xylostella* has strong reproductive ability and strong stress resistance. Currently, it has been proved that *P. xylostella* has resistance to a variety of commercial insecticides, which makes it extremely difficult to control [3]. *Tetranychus urticae* Koch (Acari: Tetranychidae) as the major miscellaneous feeding mite had the characteristics of the wide host and high reproduction rate [4,5]. In recent years, with the continuous expansion of vegetable cultivation area, the damage of *T. urticae* becomes more and more serious. The role of chemical insecticides in the management of these pests was indisputable; however, the extensive and irrational use of chemical agrochemicals has resulted in increasing resistances and environmental problems [6–8]. Cantharidin and

(S)-(-)-palasonin showed different toxicity to *P. xylostella*, with median lethal concentrations, and quinolizidine alkaloids and neem-based products exhibited moderate acaricidal activities against *T. urticae* [9–11]. Therefore, the research and development of potential pesticide alternatives from bioactive plant natural products has received much attention in recent years [12–16].

Matrine (**MT**, Figure 1) and oxymatrine (**OMT**, Figure 1) were two main alkaloids isolated from the plant *Sophora flavescens* (Kushen). Matrine was one of the promising botanical pesticides registered in China due to its good insecticidal, antibacterial, and other agricultural activities [17–20]. Oxymatrine showed a wide range of biological properties, such as pesticidal [21,22], antiviral [23], anti-inflammatory [24], and anti-tumor effects [25]. The monoterpene 1,8-cineole (**CN**, Figure 1, eucalyptol), an essential oil isolated from the eucalyptus leaves, exhibited a variety of interesting activities, such as antimicrobial [26,27], anti-inflammatory [28], and insecticidal properties [29,30]. The plant essential oils used as botanical pesticides for pest control will be a trend in the foreseeable future [31–38]. Due to their synergistic effects, a mixture of two or three bioactive compounds could play the vital role for the management of pests [39–46]. The unique advantages of plant essential oils can greatly reduce the dosage of pesticides and effectively improve their efficacy level [47,48]. Due to the problems of poor efficiency of matrine and oxymatrine, the wide application of matrine and oxymatrine as the pesticide is restricted. Therefore, in this paper, the pesticidal activities of the different complexes of 1,8-cineole with matrine and oxymatrine were investigated against *P. xylostella* and *T. urticae*. Meanwhile, detoxification enzymes in insects usually play an important role in the process of metabolizing pesticides and producing drug resistance [49]. The change in detoxification activity level may be a response to environmental stress [50]. Carboxylesterase (CarE) and glutathione *S*-transferase (GST) are two important metabolic detoxification enzymes in insects. Moreover, the epidermis, a common component of insect body walls, is an extracellular matrix released by dermal cells and plays a crucial role in maintaining insect body form, reducing water loss and resisting microbial infection and predation [51]. So, the enzyme activity changes over time of CarE and GST of treated *P. xylostella*, and the effects of compounds on the cuticles of *T. cinnabarinus* were also investigated.

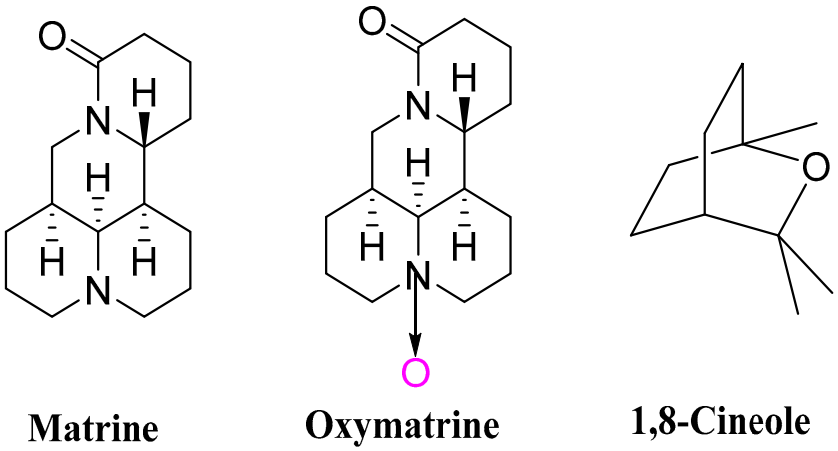

Figure 1. Chemical structures of matrine, oxymatrine and 1,8-cineole.

2. Materials and Methods

2.1. Insects and Chemicals

Tetranychus urticae (female adults) were reared for many generations in our laboratory with cowpea seedlings as the host plants to establish a stable population (temperature: 26 ± 1 °C; RH: 60–80%; photoperiod: light/dark = 14/10 h). *Plutella xylostella* (3rd instar larvae) were obtained from a laboratory population reared for many generations in the School Key Laboratory of Applied Entomology, Northwest A&F University. The cabbage net seedlings as the host plants were continuously planted in the greenhouse of our

laboratory (temperature: 25 ± 2 °C; RH: 70 ± 10%; photoperiod: light/dark = 16/8 h). The host plants did not contact any pesticides. **MT** and **OMT** (98% purity) were purchased from Baoji Haoxiang Biotechnology Co. Ltd. (Baoji, China). **CN** (99% purity) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). β-Cypermethrin (96.6% purity) was bought from Hubei Supur Chemical Co., Ltd. (Hubei, China). Spirodiclofen (98.2% purity) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

2.2. Biological Assay

2.2.1. Insecticidal Activity of Matrine (**MT**), Oxymatrine (**OMT**) and Their Binary Complexes against *P. xylostella* by the Leaf-Loading Poison Method

The solutions of matrine (**MT**), oxymatrine (**OMT**) and their binary complexes were prepared in acetone at 10 mg/mL (toosendanin as the positive control and acetone as CK) (Table 1). For each compound, 45 robust third instar larvae of *P. xylostella* were selected out (15 insects per group). The corresponding solution (1 μL) was evenly spread on a cabbage leaf disc (surface area: 0.25 cm^2). One piece of the above discs was added and eaten up by each *P. xylostella*, which was raised in each well of 12-well culture plates during 48 h (temperature: 25 ± 2 °C; RH: 70 ± 10%; photoperiod: light/dark = 16/8 h). Their corrected mortality rate (CMR) values (%) = $(T - C) \times 100/(100\% - C)$; C is the mortality rate of CK, and T is the mortality rate of the treated *P. xylostella* [52].

Table 1. Insecticidal activity of matrine (**MT**), oxymatrine (**OMT**) and their binary mixtures against *P. xylostella* at 10 μg/larva.

Compound	Corrected Mortality Rate (Mean ± SE, %)	
	24 h	48 h
MT	9.1 ± 2.2	20.9 ± 2.2 b [b]
MT/OMT (9/1) [a]	11.3 ± 0	16.2 ± 0 bc
MT/OMT (8/2)	13.6 ± 2.2	27.9 ± 2.2 a
MT/OMT (7/3)	11.3 ± 3.8	20.9 ± 2.2 b
MT/OMT (6/4)	2.3 ± 2.2	18.6 ± 2.2 bc
MT/OMT (5/5)	4.5 ± 0	20.9 ± 2.2 b
MT/OMT (4/6)	9.1 ± 2.2	18.6 ± 2.2 bc
MT/OMT (3/7)	4.5 ± 0	13.9 ± 2.2 c
MT/OMT (2/8)	2.3 ± 2.2	18.6 ± 2.2 bc
MT/OMT (1/9)	4.5 ± 0	16.2 ± 0 bc
OMT	2.3 ± 2.2	16.2 ± 0 bc
toosendanin	11.3 ± 0	32.5 ± 2.2 a

[a] Mass ratio; [b] Multiple range test using Duncan's test ($p < 0.05$). The same letters denote treatments that are not significantly different from each other.

2.2.2. Insecticidal Activity of **MT**, **OMT**, **MT**/**OMT** (8/2), 1,8-Cineole (**CN**) and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* by the Leaf-Dipping Method

The procedure for evaluation of larvicidal activity of 1,8-cineole (**CN**), **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* (Figure 2) was as follows: The solutions of 1,8-cineole (**CN**, 0.4 and 0.5 mg/mL), **MT**/**OMT** (8/2, 0.5 mg/mL), and **MT**/**OMT** (8/2, 0.5 mg/mL)/**CN** (C_{CN}= 0.4 mg/mL) were prepared in acetone (acetone was used as CK). For each compound, 45 robust third instar larvae of *P. xylostella* were selected (15 insects per group). The cabbage leaf disc (surface area: 0.25 cm^2) was dipped into the corresponding solution for 3 s and taken out. The treated ones were added to three dishes during 48 h (15 insects per dish) (temperature: 25 ± 2 °C; RH: 70 ± 10%; photoperiod: light/dark = 16/8 h). Their CMR values were calculated in the same way as mentioned above [53].

The procedure for the determination of LC_{50} values of **MT**, **OMT**, **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** at 48 h against *P. xylostella* was as follows (Table 2): Firstly, five different concentrations (2, 1, 0.5, 0.25, and 0.125 mg/mL) of **MT**, **OMT**, **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** were prepared in acetone (acetone was used as CK). Five concentrations of β-cypermethrin (a positive control) were set as 0.5, 0.25, 0.125, 0.0625,

and 0.03125 mg/mL in acetone, respectively. For each concentration, 45 robust third instar larvae of *P. xylostella* were selected (15 insects per group). The cabbage leaf disc (surface area: 0.25 cm^2) was dipped into the corresponding solution for 3 s and taken out. The treated ones were added to three dishes during 48 h (15 insects per dish) (temperature: 25 ± 2 °C; RH: 70 ± 10%; photoperiod: light/dark = 16/8 h). Their CMR values at 48 h were calculated in the same way as mentioned above. Finally, their 48 h median lethal concentration (LC_{50}) values were calculated upon the different concentrations and CMRs (Table 3).

Table 2. Corrected mortality rates at 48 h at different concentrations of **MT**, **OMT**, **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/1,8-cineole (**CN**) against *P. xylostella*.

Compound	Corrected Mortality Rate (%) at Concentration (mg/mL) [a]				
	2 (0.5) [b]	**1 (0.25)** [b]	**0.5 (0.125)** [b]	**0.25 (0.0625)** [b]	**0.125 (0.03125)** [b]
MT	65.1 ± 3.8	53.4 ± 2.2	39.5 ± 2.2	23.2 ± 0	13.9 ± 2.2
OMT	60.4 ± 4.4	48.8 ± 2.2	32.5 ± 4.4	18.6 ± 2.2	11.6 ± 2.2
MT/OMT (8/2)[c]	72.4 ± 3.3	58.6 ± 0	41.3 ± 1.6	27.5 ± 2.8	15.5 ± 1.6
MT/OMT (8/2)/**CN**[d]	81.0 ± 1.6	65.5 ± 1.6	53.4 ± 2.8	37.9 ± 0	24.1 ± 1.6
β-cypermethrin	83.7 ± 2.2	55.8 ± 5.8	37.2 ± 3.8	25.5 ± 2.2	13.9 ± 2.2

[a] Values are mean ± SE of three replicates. [b] Number in parentheses is the tested concentration of β-cypermethrin. [c] Mass ratio; [d] **MT**/**OMT** (mass ratio: 8/2) containing **CN** (C_{CN}= 0.4 mg/mL) in acetone.

Table 3. LC_{50} values of **MT**, **OMT**, **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** at 48 h against *P. xylostella*.

Compound	Linear Regression Equation[a]	LC_{50} (mg/mL)	Confidence Interval 95% (mg/mL)	r	χ^2	df	*P*	Co-Toxicity Coefficient
MT	Y = 0.057 + 1.240X	0.899	0.636~1.455	0.997	0.171	3	0.982	/
OMT	Y = −0.08 + 1.258X	1.158	0.806~2.045	0.993	0.155	3	0.984	/
MT/OMT (8/2) [b]	Y = −0.201 + 1.338X	0.708	0.539~0.970	0.998	0.025	3	0.999	133
MT/OMT (8/2)/ **CN** [c]	Y = 0.456 + 1.280X	0.441	0.324~0.585	0.999	0.161	3	0.984	213
β-cypermethrin	Y = 1.281 + 1.652X	0.168	0.128~0.226	0.980	1.845	3	0.605	/

[a] Regression analysis by IBM SPSS Statistics 20.0 ($p < 0.05$); [b] Mass ratio; [c] **MT**/**OMT** (mass ratio: 8/2) containing **CN** (C_{CN} = 0.4 mg/mL) in acetone.

The co-toxicity coefficient (CTC) values of the binary complexes were further evaluated according to Sun's formula [54]. The value of CTC is used to determine whether the efficiency is increased: when CTC > 120, it is synergistic; when CTC < 80, it is antagonistic; when 80 < CTC < 120, it is additive. A significant synergistic effect is observed when the value of CTC is 200.

2.2.3. Control Efficiency of **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* in the Greenhouse

The solutions of **MT**/**OMT** (8/2), **MT**/**OMT** (8/2)/**CN** and β-cypermethrin were prepared at 0.2 mg/mL in 0.1% aq. Tween-80, respectively (Table 4). Each cabbage seedling was infested with 20 third instar larvae of *P. xylostella* prior to spraying. One cabbage seedling was chosen for one group, and each treatment was three replicates. An airbrush was used to spray 10 mL of the corresponding solution for each treatment. The cabbage seedlings treated with 0.1% aq. Tween-80 alone were used as CK (temperature: 25 ± 2 °C; RH: 70 ± 10%; photoperiod: light/dark = 16/8 h). Their control effects on the 1st, 3rd, and 5th days were calculated in the same way as mentioned above [55].

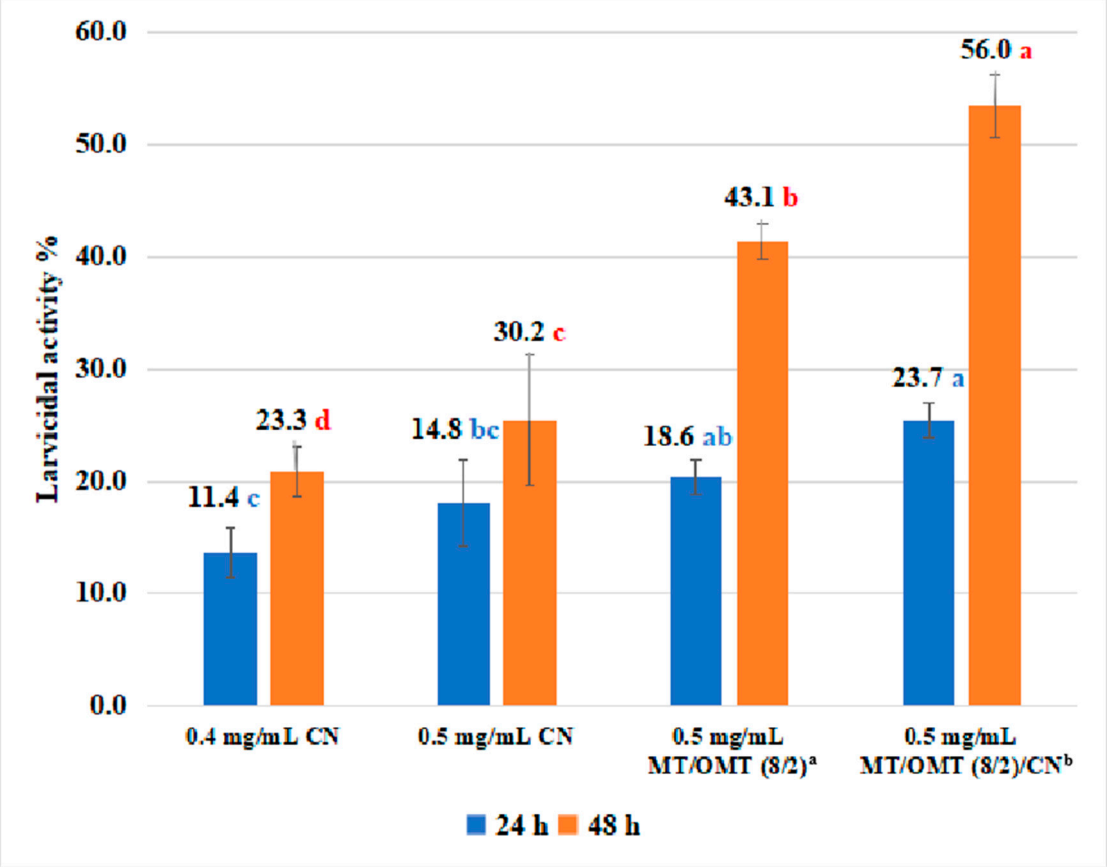

Figure 2. Larvicidal activity of 1,8-cineole (**CN**, 0.4 and 0.5 mg/mL), **MT**/**OMT** (8/2, 0.5 mg/mL) [a], and **MT**/**OMT** (8/2, 0.5 mg/mL)/**CN** [b] against *P. xylostella*. [a] Mass ratio; [b] **MT**/**OMT** (mass ratio: 8/2) were dissolved in the solution of **CN** (C_{CN}= 0.4 mg/mL) in acetone. Multiple range test using Duncan's test ($p < 0.05$). The same letters denote treatments that are not significantly different from each other (different compounds at the same time).

Table 4. Control efficiency of **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* in the greenhouse tests at a concentration of 0.2 mg/mL.

Compound	Control Efficiency (%) [c]		
	1st Day	3rd Day	5th Day
MT/OMT (8/2) [a]	10.1 ± 1.6 b [d]	25.8 ± 1.6 b	36.8 ± 0 c
MT/OMT (8/2)/**CN** [b]	15.5 ± 4.4 b	32.1 ± 3.3 b	49.0 ± 3.3 b
β-cypermethrin	40.6 ± 4.4 a	58.6 ± 2.8 a	71.9 ± 1.6 a

[a] Mass ratio; [b] **MT** and **OMT** (mass ratio: 8/2) containing **CN** (C_{CN} = 0.04 mg/mL) in 0.1% aq. Tween-80; [c] Values are mean ± SE of three replicates; [d] Multiple range test using Duncan's test ($p < 0.05$). The same letters denote treatments that are not significantly different from each other (different compounds at the same time).

2.2.4. Acaricidal Activity of **MT**, **OMT**, **CN**, and Their Mixtures against *T. urticae* by the Slide-Dipping Method

The solutions of **MT**, **OMT**, their binary complexes (with different mass ratio), and spirodiclofen (a positive control) (treated by 0.1 g/L of aq. Tween-80 as CK) were prepared at 0.5 mg/mL in Tween-80 in water (0.1 g/L), respectively (Table 5). For each compound, 90–120 healthy and size-consistency female adults of mites (30–40 ones per group) were selected out. Slides affixed with mites were dipped into the corresponding solution for 5 s and taken out (temperature: 26 ± 1 °C; RH: 60–80%; photoperiod: light/dark = 14/10 h). Their mortalities at 48 and 72 h were calculated as follows: cor-

rected mortality rate (%) = $(T - C) \times 100/(100\% - C)$; C is the mortality rate of CK, and T is the mortality rate of the treated *T. urticae* [56].

Table 5. Acaricidal activity of **MT**, **OMT**, **CN**, and their mixtures against *T. urticae* treated at a concentration of 0.5 mg/mL.

Compound	Corrected Mortality Rate (Mean ± SE, %)	
	48 h	72 h
MT	7.0 ± 2.5	23.5 ± 1.7 cde [d]
MT/OMT (9/1) [a]	10.5 ± 1.6	20.4 ± 3.2 cdef
MT/OMT (8/2)	6.9 ± 0.7	24.3 ± 2.3 cde
MT/OMT (7/3)	4.5 ± 1.0	21.9 ± 5.8 cdef
MT/OMT (6/4)	10.3 ± 0.5	16.0 ± 3.4 ef
MT/OMT (5/5)	10.9 ± 1.6	24.6 ± 4.4 cde
MT/OMT (4/6)	8.7 ± 2.0	27.1 ± 3.4 bcde
MT/OMT (3/7)	9.4 ± 3.2	32.2 ± 0.9 bc
MT/OMT (2/8)	9.8 ± 2.2	28.9 ± 1.6 bcd
MT/OMT (1/9)	4.0 ± 1.2	25.2 ± 6.3 cde
OMT	6.0 ± 1.3	18.2 ± 2.9 def
1,8-cineole (CN) [b]	6.5 ± 0.2	11.2 ± 0.4 f
MT/OMT (3/7)/**CN** [c]	17.8 ± 0.3	38.0 ± 3.2 b
spirodiclofen	47.7 ± 4.7	88.6 ± 2.3 a

[a] Mass ratio; [b] at a concentration of 0.048 mg/mL; [c] **MT**/**OMT** (mass ratio: 3/7) containing **CN** (C_{CN}= 0.048 mg/mL) in Tween-80 in water (0.1 g/L); [d] Multiple range test using Duncan's test ($p < 0.05$). The same letters denote treatments that are not significantly different from each other (different compounds at the same time).

According to the above results, the acaricidal activity of **CN** and **MT**/**OMT** (3/7)/**CN** against *T. urticae* (Table 5) was tested as follows: The solution of **CN** was prepared at 0.048 mg/mL in Tween-80 in water (0.1 g/L), and the solution of **MT**/**OMT** (3/7)/**CN** was prepared at 0.5 mg/mL in Tween-80 in water (0.1 g/L) containing **CN** (C_{CN}= 0.048 mg/mL). The next procedure was completed in the same way as mentioned above.

2.2.5. LC_{50} of **MT**, **OMT**, **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** against *T. urticae*

Firstly, five different concentrations (6, 3, 1.5, 0.75, and 0.375 mg/mL) of **MT** and **OMT** were prepared in Tween-80 in water (0.1 g/L) (treated by 0.1 g/L of aq. Tween-80 as CK) (Table 6). Six concentrations of **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** were set as 4, 2, 1, 0.5, 0.25, and 0.125 mg/mL in Tween-80 in water (0.1 g/L), respectively. Five concentrations of spirodiclofen were set as 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL in Tween-80 in water (0.1 g/L), respectively. For each concentration, 90–120 healthy and size-consistency female adults of mites (30–40 ones per group) were selected out. Slides affixed with mites were dipped into the corresponding solution for 5 s and taken out (temperature: 26 ± 1 °C; RH: 60–80%; photoperiod: light/dark = 14/10 h). Their CMR values at 72 h were calculated in the same way as mentioned above. Finally, LC_{50} values were calculated by the linear regressions of 72 h CMRs (%) and concentrations (Table 7) [56]. The co-toxicity coefficient (CTC) values of the binary complexes were further evaluated according to Sun's formula [54].

2.2.6. Control Efficiency of **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** against *T. urticae* in the Greenhouse

The solutions of **MT**/**OMT** (3/7), **MT**/**OMT** (3/7)/**CN**, and spirodiclofen were all prepared at 0.2 mg/mL in 0.1% aq. Tween-80, respectively. Asparagus bean plants were infested with the female adults of *T. urticae* prior to spraying. Three plants were chosen for one group, and each treatment was three replicates. An airbrush was used to spray 10 mL of the corresponding solution for three replicates. The plants treated with 0.1% aq. Tween-80 alone were used as CK (temperature: 26 ± 1 °C; RH: 60–80%; photoperiod:

light/dark = 14/10 h). Their control effects on the 1st, 3rd, and 5th days were calculated in the same way as mentioned above (Table 8) [56].

Table 6. Corrected mortality rates at 72 h at different concentrations of **MT**, **OMT**, **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** against *T. urticae*.

Compound	Corrected Mortality Rate (%) at Concentration (mg/mL) [a]					
	6	**3**	**1.5**	**0.75**	**0.375**	**/**
MT	80.3 ± 0.9	56.6 ± 1.6	40.5 ± 2.4	24.1 ± 2.7	15.6 ± 3.9	/
OMT	75.1 ± 2.4	53.9 ± 2.2	38.5 ± 2.1	20.5 ± 3.5	10.3 ± 1.0	/
Compound	Corrected mortality rate (%) at concentration (mg/mL) [a]					
	4 (0.5) [b]	2 (0.25) [b]	1 (0.125) [b]	0.5 (0.0625) [b]	0.25 (0.03125) [b]	0.125
MT/OMT (3/7) [c]	72.6 ± 0.4	53.2 ± 1.3	40.4 ± 2.7	32.2 ± 0.9	21.7 ± 0.6	13.4 ± 1.5
MT/OMT (3/7)/**CN** [d]	80.1 ± 0.8	61.0 ± 0.5	49.6 ± 2.2	38.0 ± 3.2	29.6 ± 3.5	17.4 ± 1.6
spirodiclofen	90.1 ± 0.5	72.5 ± 0.2	54.5 ± 0.8	36.8 ± 2.6	26.0 ± 1.2	/

[a] Values are mean ± SE of three replicates. [b] Number in parentheses is the tested concentration of spirodiclofen. [c] Mass ratio; [d] **MT** and **OMT** (mass ratio: 3/7) containing **CN** (C_{CN} = 0.048 mg/mL) in Tween-80 in water (0.1 g/L).

Table 7. LC_{50} values of **MT**, **OMT**, **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** at 72 h against *T. urticae*.

Compound	Linear Regression Equation [a]	LC_{50} (mg/mL)	Confidence Interval 95% (mg/mL)	r	χ^2	df	*P*	Co-Toxicity Coefficient
MT	Y = −0.471 + 1.549X	2.014	1.667~2.451	0.987	2.004	3	0.572	/
OMT	Y = −0.604 + 1.606X	2.377	1.970~2.924	0.994	0.398	3	0.941	/
MT/OMT (3/7) [b]	Y = −0.160 + 1.083X	1.405	1.108~1.858	0.986	1.681	4	0.794	160
MT/OMT (3/7)/**CN** [c]	Y = 0.054 + 1.103X	0.894	0.713~1.134	0.992	2.166	4	0.705	252
spirodiclofen	Y = 1.597 + 1.558X	0.094	0.077~0.113	0.996	2.198	3	0.532	/

[a] Regression analysis by IBM SPSS Statistics 20.0 ($p < 0.05$); [b] Mass ratio; [c] **MT** and **OMT** (mass ratio: 3/7) containing **CN** (C_{CN} = 0.048 mg/mL) in Tween-80 in water (0.1 g/L).

Table 8. Control efficiency of **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** against *T. urticae* in the greenhouse tests at a concentration of 0.2 mg/mL.

Compound	Control Efficiency (%) [c]		
	1st Day	**3rd Day**	**5th Day**
MT/OMT (3/7) [a]	7.7 ± 0.8 b [d]	19.6 ± 1.9 c	31.3 ± 1.1 c
MT/OMT (3/7)/**CN** [b]	13.8 ± 1.8 b	31.9 ± 1.0 b	42.3 ± 1.2 b
spirodiclofen	25.0 ± 2.4 a	58.3 ± 1.0 a	73.9 ± 1.5 a

[a] Mass ratio; [b] **MT** and **OMT** (mass ratio: 3/7) containing **CN** (C_{CN} = 0.04 mg/mL) in 0.1% aq. Tween-80; [c] Values are mean ± SE of three replicates; [d] Multiple range test using Duncan's test ($p < 0.05$). The same letters denote treatments that are not significantly different from each other (different compounds at the same time).

2.3. Enzyme Activity Assay against P. xylostella

2.3.1. Sample Preparation Using Leaf-Dipping Method

According to the above-mentioned leaf-dipping method, 180 robust 3rd instar larvae of *P. xylostella* were treated with **CN**, **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** at 0.4 mg/mL, respectively (treated by acetone as CK). Then, the 30 surviving larvae in the treated group were collected at 12, 24, 36, and 48 h, respectively. They were then snap-frozen in liquid nitrogen and stored at −80 °C for subsequent enzyme activity analysis [53].

2.3.2. Preparation of Homogenous Liquid

The homogenization treatment (including ten larvae: Weight (g)/Volume (mL) = 1/10) was performed in an ice bath. The homogenous liquid was obtained for carboxylesterase

(CarE) activity assay when the sample was centrifuged at 12,000× *g* for 30 min at 4 °C. The homogenous liquid was obtained for glutathione-*S*-transferase (GST) activity assay when the sample was centrifuged at 8000× *g* for 10 min at 4 °C.

2.3.3. CarE and GST Activity Assay According to Bradford's Method

The absorption values over time of CarE and GST were tested by using CarE (α-naphthyl acetate (α-NA) as a substrate) and GST (1-chloro-2,4-dinitrobenzene and reduced glutathione as substrates) assay kits (Suzhou Keming Biotechnology Co., Ltd., China), respectively. Total protein concentration was determined according to the Bradford method (using bovine serum albumin (BSA) as a standard). The protein content was tested by a BCA protein quantitative assay kit (Shaanxi Zhonghui Hecai Biomedical Technology Co., Ltd., China). Finally, the enzymes activity values were obtained according to the absorption value and the protein content. Each treatment was replicated three times [57].

2.4. Analysis of Morphology of Cuticles

2.4.1. Pretreatment of Mites

The complex (**MT**/**OMT** (3/7)/**CN**) was prepared at 0.894 mg/mL in 0.1 g/L of aq. Tween-80 (0.1 g/L of aq. Tween-80 as CK). Female adult mites with the same physiological status and good growth conditions were selected to cowpea leaves (5 cm in length and 3 cm in width; 30 ones/leaf). After 4 h, the leaves were immersed in the above solution for 5 s, the excess solution was absorbed by filter paper, and the leaves were placed in a Petri dish with a moist sponge (three replicates/treatment). Then, Petri dishes were placed in a light incubator (temperature: 26 ± 1 °C; RH: 60–80%; photoperiod: light/dark = 14/10 h). Finally, the dead mites were collected at 24, 48 and 72 h after treatment by the complex for scanning electron microscope (SEM) analysis [56,58].

2.4.2. Observation of Morphology and Structural Changes of Cuticles in Spider Mites

The collected samples were fixed with 2.5% glutaraldehyde under ice bath conditions, incubated at 4 °C for 4 h, rinsed three times with 0.1 mol/L of phosphate buffer saline (PBS), dehydrated with different concentrations of ethanol, and freeze-dried for 3 h. The mites were then placed on a sample table and sprayed with gold under vacuum conditions. The morphology was observed and photographed by S-3400N SEM [59].

2.5. Statistic Analysis

Mortality data were corrected with Abbott's formula and analyzed by a multiple range test using Duncan's test ($p < 0.05$). The median lethal concentration (LC_{50}) values were calculated on log-concentration versus probit (% mortality) regression analysis. The values of r, χ^2, df, and *P* were obtained on regression analysis by IBM SPSS Statistics 20.0 ($p < 0.05$).

3. Results and Discussion

3.1. Insecticidal Activity

First, the insecticidal activities of two compounds (**MT** and **OMT**) and their binary mixtures at different mass ratios against *P. xylostella* were tested. As shown in Table 1, to binary mixtures, when the mass ratio of **MT** and **OMT** was 8:2, the corresponding 48 h corrected mortality rate (CMR) was 27.9%, which was higher than those of **MT** (20.9%) and **OMT** (16.2%). The 48 h CMRs were all 20.9% when the mass ratio of **MT**/**OMT** was 7:3 or 5:5. The 48 h CMR was 16.2% when the mass ratio of **MT**/**OMT** was 1:9, which was similar to that of **OMT**. However, when the mass ratio of **MT** and **OMT** was 3:7, the corresponding 48 h CMR was decreased to 13.9%, which was lower than those of **MT** and **OMT**. Obviously, for **MT** and **OMT** against *P. xylostella*, the best mass ratio of **MT** and **OMT** was 8:2. As shown in Figure 2, against *P. xylostella*, 48 h CMRs of 1,8-cineole (**CN**, 0.4 and 0.5 mg/mL), **MT**/**OMT** (8/2, 0.5 mg/mL), and **MT**/**OMT** (8/2, 0.5 mg/mL)/**CN** were 23.3%, 30.2%, 43.1%, and 56.0%, respectively. It demonstrated that when **CN** (0.4 mg/mL) was combined with **MT**/**OMT**(8/2, 0.5 mg/mL), the larvicidal activity of the corresponding complex

was significantly improved. Then, 48 h LC_{50} values of **MT**, **OMT**, **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* were further evaluated according to their CMRs at different concentrations (Table 2). As described in Table 3, 48 h LC_{50} values of **MT**/**OMT** (8/2), and **MT**/**OMT** (8/2)/**CN** were 0.708 and 0.441 mg/mL, respectively; while 48 h LC_{50} values of **MT** and **OMT** were 0.899 and 1.158 mg/mL, respectively. That is, the larvicidal activity of **MT**/**OMT** (8/2)/**CN** was 2.0–2.6 fold those of **MT** and **OMT**. Moreover, the co-toxicity coefficient (CTC) values of **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** were 133 and 213, respectively. Clearly, their CTCs were all > 120, so the above two mixtures showed the synergistic effect. Subsequently, the control effects of **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* in the greenhouse at 0.2 mg/mL were tested. As shown in Table 4, the control effects of **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* after 5 days were 36.8% and 49.0%, respectively. Symptoms at the fifth day of the cabbage seedling leaves treated with **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** against *P. xylostella* are described in Figure 3. In the **MT**/**OMT** (8/2)- and **MT**/**OMT** (8/2)/**CN**-treated groups, the surfaces of the leaves were smooth and had almost no small puncture; however, there were many small punctures eaten by *P. xylostella* on the leaves in the CK-treated group. Photographs of control effects of those complexes against *P xylostella* are shown in Figures S1–S3.

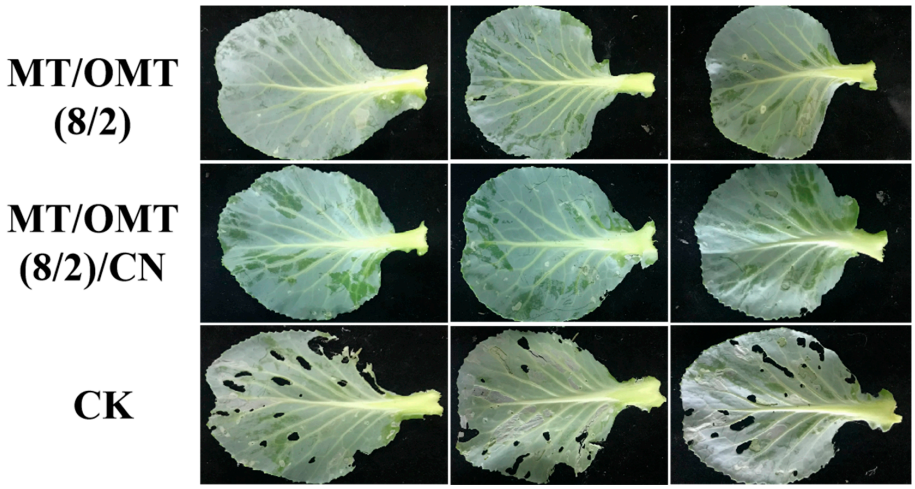

Figure 3. Fifth-day symptoms of cabbage seedling leaves treated with **MT**/**OMT** (8/2, 0.2 mg/mL) and **MT**/**OMT** (8/2, 0.2 mg/mL)/**CN** against *P. xylostella*.

3.2. Acaricidal Activity

The 48 and 72 h acaricidal results of **MT**, **OMT**, and their binary mixtures at 0.5 mg/mL against the female adults of *T. urticae* are shown in Table 5. Among nine binary complexes, the 72 h CMRs of six complexes were higher than those of **MT** (23.5%) and **OMT** (18.2%); especially when the mass ratio of **MT** to **OMT** was 3:7, the corresponding 72 h CMR was 34.8%. At 0.048 mg/mL, the 72 h CMR of **CN** against *T. urticae* was only 11.2%; interestingly, when **MT** and **OMT** (mass ratio: 3/7) were dissolved in the solution of **CN** ($C_{\mathbf{CN}}$ = 0.048 mg/mL) in 0.01% aq. Tween-80, and $C_{\mathbf{MT/OMT}\ (3/7)}$ was 0.5 mg/mL, the corresponding CMR was increased to 38.0%. It may be related to the strong permeability of **CN** as an essential oil [38]. Then, 72 h LC_{50} values of **MT**, **OMT**, **MT**/**OMT** (3/7), and **MT**/**OMT** (3/7)/**CN** against *T. urticae* were further calculated according to their CMRs at different concentrations (Table 6). As shown in Table 7, the 72 h LC_{50} values of **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** ($C_{\mathbf{CN}}$= 0.048 mg/mL) were 1.405 and 0.894 mg/mL, respectively, while 72 h LC_{50} values of **MT** and **OMT** were 2.014 and 2.377 mg/mL, respectively. That is, the acaricidal activity of **MT**/**OMT** (3/7)/**CN** ($C_{\mathbf{CN}}$= 0.048 mg/mL) was increased to 2.3–2.7 folds when compared with those of **MT** and **OMT**. Meanwhile, the CTCs of **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** ($C_{\mathbf{CN}}$= 0.048 mg/mL) were 160 and 252, respectively. So, the above two mixtures showed the synergistic effect especially

for the combination of **MT**/**OMT** (3/7)/**CN**. On the other hand, the control efficiency of **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** (C_{CN} = 0.04 mg/mL in 0.1% aq. Tween-80) at 0.2 mg/mL against *T. urticae* in the greenhouse was evaluated. As shown in Table 8, the control effects of **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** after 5 days were 31.3% and 42.3%, respectively. Fifth-day symptoms of asparagus bean seedling leaves treated with **MT**/**OMT** (3/7) and **MT**/**OMT** (3/7)/**CN** were described in Figure 4. There were lots of white spots destroyed by *T. urticae* on the seedling leaves in the CK-treated group, whereas in the **MT**/**OMT** (3/7)- and **MT**/**OMT** (3/7)/**CN**-treated groups, almost no small white spots were on the seedling leaves. These findings were the same as our previous report [58]. Photographs of control effects of those complexes against *T. urticae* are shown in Figures S4–S6.

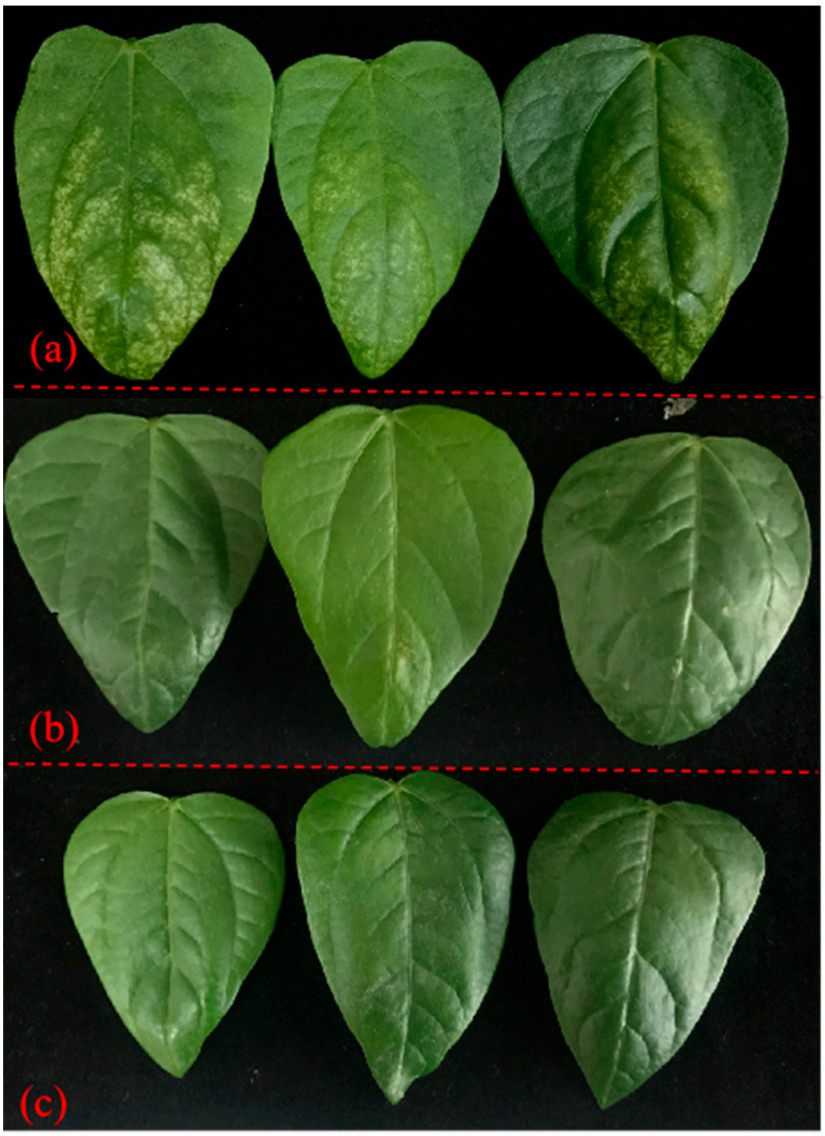

Figure 4. Fifth-day symptoms of asparagus bean seedling leaves treated with **CK** (**a**), **MT**/**OMT** (3/7, 0.2 mg/mL) (**b**), and **MT**/**OMT** (3/7, 0.2 mg/mL)/**CN** (**c**) against *T. urticae*.

3.3. Changes of Detoxification Enzymes Activities

Subsequently, the changes of detoxification enzymes (CarE and GST) activities in *P. xylostella*, treated with **CN**, **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** at 0.4 mg/mL after 12, 24, 36, and 48 h were depicted in Figures 5 and 6, respectively. The enzymatic activities of CarE and GST are always in dynamic change. As described in Figure 5, the CarE activity

values in the treated groups at 36 and 48 h were much lower than those of the control group. The CarE activity value in the **CN**-treated group at 12 h (82.1 U/g) was higher than that of the control group (69.4 U/g). It suggested that **CN** was toxic to *P. xylostella* and stimulated the detoxification ability of the CarE in *P. xylostella* at 12 h. At 24 h, the CarE activity value in the **MT**/**OMT** (8/2)/**CN**-treated group (67.7 U/g) was higher than that of the control group (52.4 U/g). It indicated that **MT**/**OMT** (8/2)/**CN** stimulated the detoxification ability of the CarE in *P. xylostella* at 24 h. After that, the detoxification ability of the CarE in *P. xylostella* in all treated groups was inhibited. The time for reaching the lowest points of the CarE activities in the treated groups was different. For example, in the **CN**-treated group, the lowest point of the CarE activity was reached at 36 h, whereas the lowest points of the CarE activity in the **MT**/**OMT** (8/2)- and **MT**/**OMT** (8/2)/**CN**-treated group were reached at 48 h.

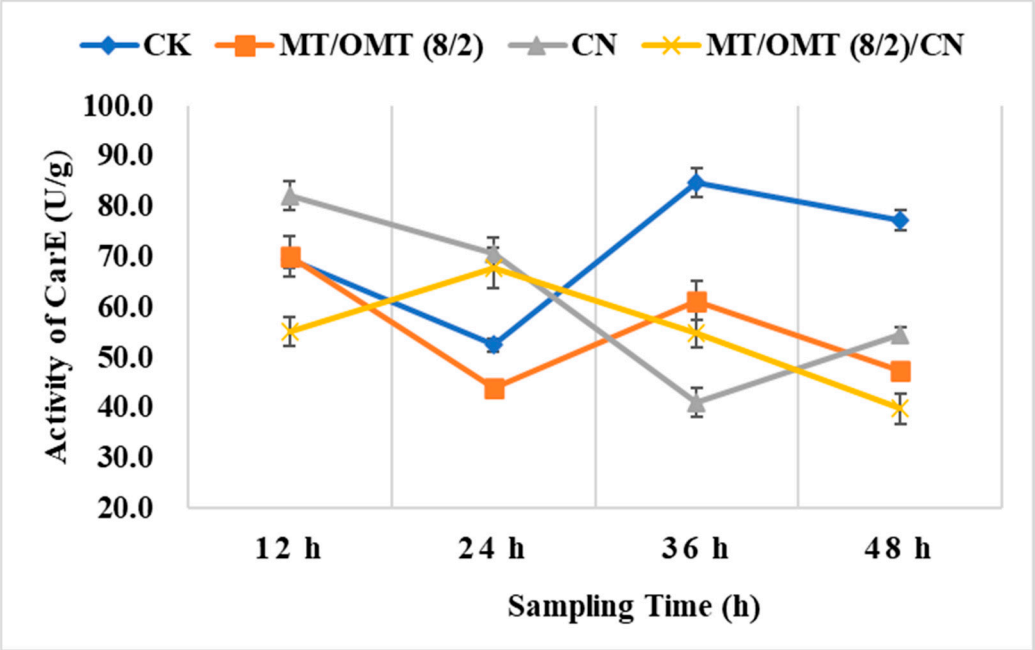

Figure 5. Effects of **CN**, **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** at 0.4 mg/mL on CarE in *P. xylostella* at different sampling times..

As illustrated in Figure 6, the GST activity value in the **MT**/**OMT** (8/2)/**CN**-treated group at 12 h (3910 nmol/min/g) was higher than that of the control group (2698 nmol/min/g). Similarly, it suggested that the mixture **MT**/**OMT** (8/2)/**CN** stimulated and enhanced the detoxification ability of the GST in *P. xylostella* at 12 h. At 24 h, the GST activity value in the **CN**-treated group was slightly higher than that of the control group. Afterwards, the GST activity in the treated groups decreased significantly when compared with that of the control, and it reached the lowest point at 36 h in all treated groups. The GST activity values at 36 h in **CN**-, **MT**/**OMT** (8/2)- and **MT**/**OMT** (8/2)/**CN**-treated groups were 2208, 2591 and 1932 nmol/min/g, respectively, and they were decreased 1.4–1.9 folds of that of the control group (3603 nmol/min/g). Obviously, the GST activity of *P. xylostella* in the treated groups was largely inhibited at 36 h.

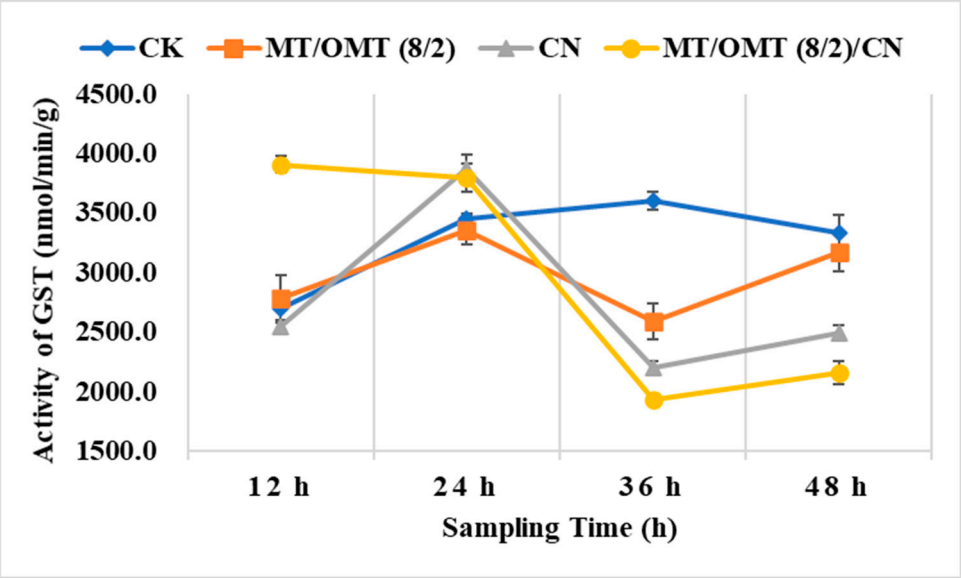

Figure 6. Effects of **CN**, **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** at 0.4 mg/mL on GST in *P. xylostella* at different sampling times.

3.4. Toxicological Study of Structural Changes of Cuticles by SEM

The penetration of insecticide to pest epidermis is the premise of its toxic effect. The cuticle serves as the initial barrier of defense between the body and the outside world, effectively blocking pesticide penetration. The reduction in epidermal penetration can delay the time of pesticide reaching the target site, and pests have sufficient time and faster speed to metabolize the pesticide entering the body [59]. As shown in Figure 7, in the CK-treated group, the cuticles of *T. urticae* are flat and have an entire structure, with neat and continuous skin texture; whereas in the **MT/OMT** (3/7)**/CN**-treated group, the structure of the cuticles was damaged with visible wrinkles and an uneven arrangement of the inner ridges. The cuticles of wounded mites were thinner, softer, and more asymmetrically bent than those of normal mites, losing their barrier-like function against acaricide penetration. As a result, **CN** may enhance the penetration ability of this complex on *T. urticae* [38,60].

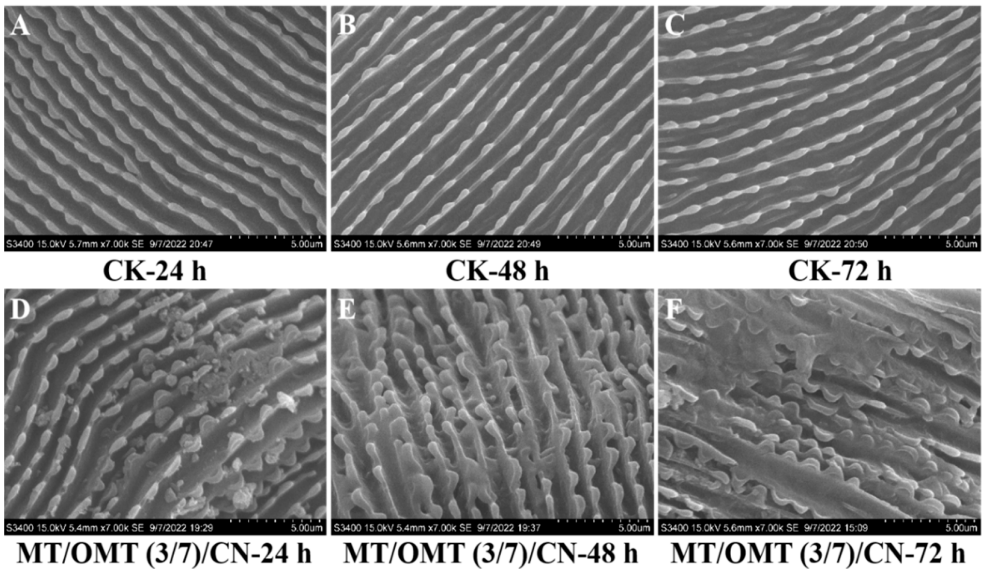

Figure 7. Scanning electron microscope images of cuticles of *T. urticae* treated with **MT/OMT** (3/7)**/CN** (**D**–**F**) and aq. Tween-80 (0.1 g/L) (CK) (**A**–**C**) after 24, 48 and 72 h, respectively. Bars: 5.0 μm.

4. Conclusions

In summary, the significant synergistic effects were observed when **CN** was combined with **MT** and **OMT** as pesticidal agents: against *P. xylostella*, the CTC of **MT**/**OMT** (8/2)/**CN** was 213; against *T. urticae*, the CTC of **MT**/**OMT** (3/7)/**CN** was 252. Furthermore, these mixtures displayed good control efficiency against *P. xylostella* and *T. urticae* in the greenhouse. Importantly, the enzymes activity changes over time of CarE and GST in *P. xylostella* treated with **CN**, **MT**/**OMT** (8/2) and **MT**/**OMT** (8/2)/**CN** were explored. Due to the accumulation of toxicant leading to a decrease in the detoxification ability, the time for reaching the lowest point of the CarE activity in the treated groups was different, whereas the time for reaching the lowest point of the GST activity in the treated groups was the same (at 36 h). Notably, by SEM analysis, the toxicology study suggested that the destruction of the cuticle layer crest of *T. urticae* by **MT**/**OMT** (3/7)/**CN** may be the main cause of their death. These results will pave the way for the future study of different combinations of **MT**–**OMT**–**CN** and the application of **CN** as a synergist with other bioactive natural products as pesticidal candidates in crop protection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins15040240/s1, Biological assay; Pictures of control effects of different complexes against *P. xylostella*; Pictures of control effects of different complexes against *T. urticae*. Figure S1. Pictures of control efficiency of **CK** after 1st day (a), 3rd day (b) and 5th day (c) against *P. xylostella* in the greenhouse; Figure S2. Pictures of control efficiency of **MT**/**OMT** (8/2, 0.2 mg/mL) after 1st day (a), 3rd day (b) and 5th day (c) against *P. xylostella* in the greenhouse; Figure S3. Pictures of control efficiency of **MT**/**OMT** (8/2, 0.2 mg/mL)/**CN** after 1st day (a), 3rd day (b) and 5th day (c) against *P. xylostella* in the greenhouse; Figure S4. Pictures of control efficiency of **CK** after 1st day (a), 3rd day (b) and 5th day (c) against *T. urticae* in the greenhouse; Figure S5. Pictures of control efficiency of **MT**/**OMT** (3/7, 0.2 mg/mL) after 1st day (a), 3rd day (b) and 5th day (c) against *T. urticae* in the greenhouse; Figure S6. Pictures of control efficiency of **MT**/**OMT** (3/7, 0.2 mg/mL)/**CN** after 1st day (a), 3rd day (b) and 5th day (c) against *T. urticae* in the greenhouse.

Author Contributions: J.X., S.F., Y.W., H.W. and S.Z. who have made substantial contributions to the present work are named in the manuscript. M.L. and H.X. designed the work, analyzed the data, and wrote and revised the paper. All authors have read and agreed to the published version of the manuscript.

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