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Comparative studies on muscle microstructure and ultrastructure of *Mythimna separata* Walker treated with wilforgine and chlorantraniliprole

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ABSTRACT

We attempted to elucidate the comparative effects between wilforgine and chlorantraniliprole on the microstructure/ultrastructure of muscle tissue in *Mythimna separate* larvae. The typical toxicity symptoms of *M. separata* larvae upon wilforgine treatment was feeding cessation and flaccid paralysis, whereas feeding cessation and contraction paralysis were the main poisoning symptoms wrought by chlorantraniliprole. Light-microscopy observations showed that the microstructure of muscle tissue could be damaged by wilforgine and chlorantraniliprole, and the death of insects was associated with muscle lesions. Muscle tissue was loose after wilforgine treatment but constricted muscle tissue was observed upon chlorantraniliprole treatment. Transmission electron microscopy showed that wilforgine and chlorantraniliprole could disrupt endomembranes and plasma membranes. These results suggest that wilforgine can induce microstructural and ultrastructural changes in the muscles of *M. separata* larvae; the sites of action are proposed to be calcium receptors or channels in the muscular system.

1. Introduction

For many years, protection of agricultural crops has been based mainly on synthetic pesticides (Ponsankar et al., 2016). However, the long-term use of synthetic pesticides, especially non-selective pesticides, has evoked environmental pollution, risks to human health, pest resistance, and direct toxicity to non-target organisms (Maheswaran and Ignacimuthu, 2013; Thanigaivel et al., 2017). Therefore, selective pesticides are the mainstream of research (Banks et al., 2008). Selective pesticides are more desirable than non-selective pesticides, because their ecological impact is potentially much smaller (Stark and Banks, 2001). In recent years, in view of high selectivity, biodegradability and ecological friendliness (Isman, 2015; Pino et al., 2015; Qi et al., 2011; Wu et al., 2014), botanical pesticides have been paid to great attention. Moreover, new mechanisms of action (MoAs) have been discovered through the research and development of botanical pesticides (El-Wakeil, 2013; Gross, 2014; Joshi et al., 2014; Mehlhorn et al., 2011).

Tripterygium wilfordii Hook f. (Celastraceae), known as *lei gong teng* (“thunder God vine”) in China, has been used as a traditional Chinese medicine (TCM) for the treatment of cancer, fever, autoimmune diseases, joint pain, and skin disorders for two decades (Lü et al., 2015; Qiu and Kao, 2003; Mao and Huang, 2016). Preparations of its root bark began to be used as a homemade insecticide to control pests on smallholder properties in China in the 1990s (Chiu and Qiu, 1993). Previous studies have demonstrated that the main insecticidal ingredients in *T. wilfordii* are alkaloids (Luo et al., 2000, 2004; Ma et al., 2014), and the alkaloid-based products are safe to non-target animals (Li et al., 2012; Ma et al., 2007; Wang et al., 2012). However, the MoA of this insecticide is not clear, which greatly limits the further development and application of *T. wilfordii* insecticide in organic agriculture. Thus, it is necessary to elucidate its insecticidal mechanisms.

Preliminary observations have suggested that *Mythimna separata* larvae are paralyzed by the total alkaloids of *T. wilfordii*; the structure of larvae muscle cells were damaged and insect death was associated with muscle lesions according to histopathology (Zhou, 2007). The special symptoms associated with toxicity mean that the insecticidal mechanism of the total alkaloids of *T. wilfordii* likely differs from that of conventional pesticides, which typically affect the function of the γ-aminobutyric acid receptor, mitochondrial respiration, acetylcholine-receptor function, chitin synthesis, or sodium-channel function (Dekeyser, 2005; Narahashi, 2002; Nauen and Bretschneider, 2002). Wilforgine (Fig. 1), with similar toxicity symptoms to total alkaloids, may also affect the muscular system of insects. Interestingly, according to the IRAC mode of action classification scheme (version 8.1) (IRAC, 2016), only diamides mainly act on muscle system. Chlorantraniliprole can act on ryanodine receptors, which can mediate the release of

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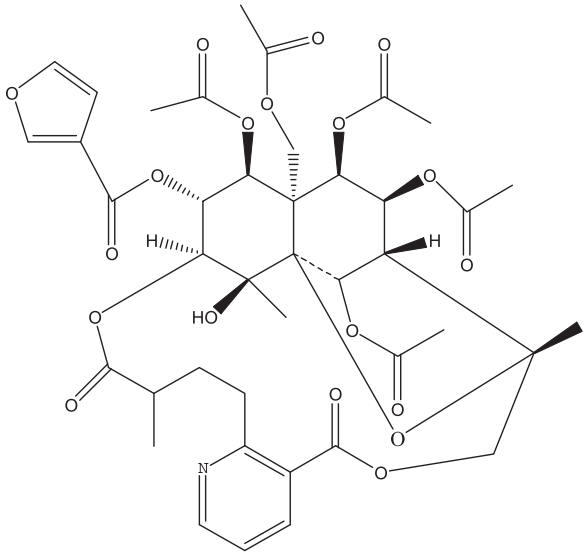

Fig. 1. Structure of wilforgine.

calcium ions (Ca^{2+}) from intracellular stores of the sarcoplasmic reticulum (SR) and endoplasmic reticulum, and cause the contraction and paralysis of muscle (Cordova et al., 2006; Lahm et al., 2009).

We compared the effects of wilforgine and chlorantraniliprole on the microstructure/ultrastructure of muscles from *M. separata* Walker larvae to improve understanding of the histopathological MoA induced by wilforgine.

2. Material and methods

2.1. Insects and chemicals

A laboratory-adapted strain of *M. separata* was provided by the Research & Development Center of Biorational Pesticides, Northwest A & F University (Yangling, China). A *M. separata* colony was reared in an insectary at controlled temperature (25 ± 1 °C), relative humidity (75 ± 5%) and a fixed photoperiod (14 h of light and 10 h of darkness).

Wilforgine (> 98%) was obtained from the Research & Development Center of Biorational Pesticides. Chlorantraniliprole (> 95%) was purchased from DuPont Agricultural Chemicals (Shenzhen, China).

2.2. Treatment of insects

The leaf disk method was used for this bioassay (Satasook et al., 1992). Based on the results of bioassays, the LC_{80} values of wilforgine and chlorantraniliprole on fifth-instar larvae of *M. separate* were determined to be about 200.0 μg/mL and 2.0 μg/mL, respectively. Firstly, thirty one-day-old fifth-instar larvae of *M. separate* were placed individually into a single Petri dish (diameter, 6 cm), and then starved for 12 h. Subsequently, fresh corn leaf disks (1 cm × 1 cm) were cut and immersed in wilforgine acetone solution (200.0 μg/mL) and chlorantraniliprole acetone solution (2.0 μg/mL) for 3 s. Negative control disks were immersed in acetone solution. After evaporation of the solvent, treated leaf disks were supplied to each larva, and the symptoms of poisoning were recorded over the next 2–3 days, until death. Larvae at different stages of poisoning were selected for light microscopy (LM) and transmission electron microscopy (TEM) observations.

2.3. LM

The second-to-sixth internodes of the somatic muscle of *M. separata* larvae were dissected carefully, and then fixed quickly in Bouin's solution (saturated picric acid:formaldehyde:acetic acid = 75:25:5) for 24 h. Subsequently, the specimens were washed twice in 70% ethanol solution and dehydrated in an ascending series of ethanol solutions (30%, 50%, 70%, 80%, 90%, 100%, *v/v*). Finally, the samples were prepared by a traditional method (Lü et al., 2010). Thick sections were cut with a glass knife and stained with hematoxylin & eosin, and the prepared samples photographed under a light microscope (Nikon, Tokyo, Japan).

2.4. TEM

Muscle samples were cut into thin pieces (≈ 1 mm^3) and placed in 2.5% glutaraldehyde at 4 °C for 4 h. Subsequently, thin pieces were rinsed with 0.1 M phosphate buffer (pH 7.2) for 5, 10, 15, 20, or 30 min, respectively. Specimens were post-fixed in 1% osmium tetroxide for 2 h at 4 °C and rinsed again with 0.1 M phosphate buffer. Muscles were dehydrated in an acetone series in ascending order (30%, 50%, 70%, 80%, 90% and 95%, *v/v*) for 15-min each and infiltrated in a mixture of LR-White resin and alcohol (1:1, *v/v*). After polymerization for 48 h at room temperature, ultrathin sections were cut using a diamond knife on an EM UC7 ultramicrotome (Leica, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. Finally, the prepared sections were photographed under a transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV.

3. Results

3.1. Symptomology of poisoning

M. separata larvae displayed a series of toxicity symptoms after wilforgine administration. During a “preliminary poisoning” stage at 3 h, the larvae could not move, adhered to the bottom of petri dishes, stopped feeding and dully responded to gentle stimulation: this stage was defined as “weak paralysis” (Fig. 2B); the control larvae could feed and move, and responded to gentle stimulation sensitively (Fig. 2A). About 8-h later, some of the wilforgine-treated larvae began to lie along the side of the petri dish, and did not respond to gentle stimulation, but the trembling feet of larvae could be observed under an anatomic lens: this stage was called “moderate paralysis” (Fig. 2C). The wilforgine-treated larvae began to enter a state of “deep paralysis” 20 h after treatment. The relaxed inter-segmental membranes of the larvae could be observed obviously, and the wilforgine-treated larvae could be folded across the middle with a pin (Fig. 2D).

The toxicity symptoms of chlorantraniliprole-treated *M. separata* larvae were also recorded. During a preliminary-poisoning stage at 1–2 h, the larvae stopped feeding, and movements were very restricted and extremely slow (Fig. 2F); the control larvae could feed and move (Fig. 2E). After eating the treated leaves for 4–6 h, the chlorantraniliprole-treated larvae could not move away, and exhibited a gradual contraction paralysis of the whole body while adopting an irregular C-hook posture at the moderate-poisoning stage (Fig. 2G). Finally, the larvae entered the late-poisoning stage: chlorantraniliprole caused

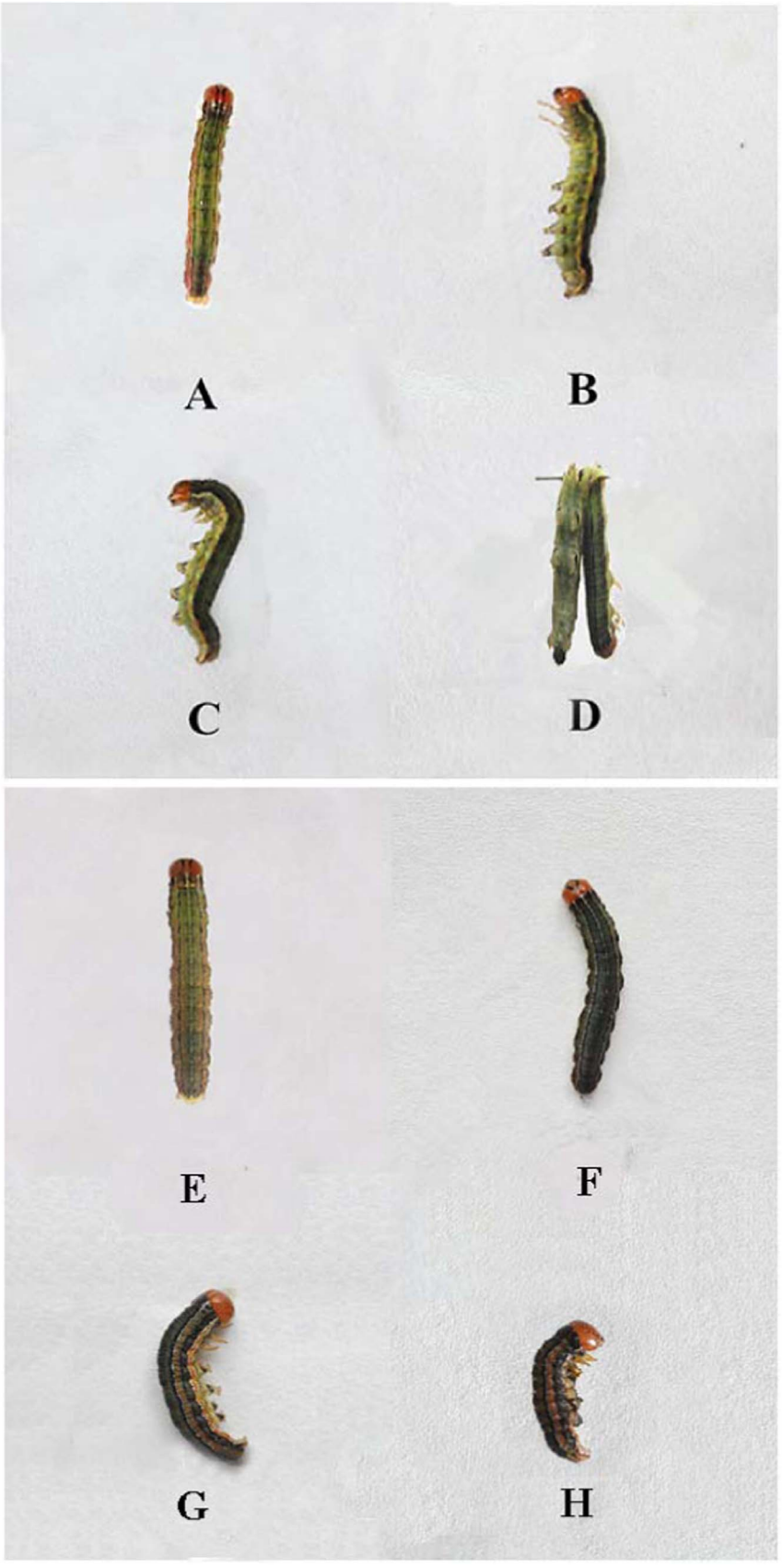

Fig. 2. Symptoms of fifth-instar larvae of *M. separata* treated with wilforgine and chlorantraniliprole. (A and E) Control larvae. Wilforgine-treated larvae: (B) Poisoned larvae in the weak paralysis stage. (C) Poisoned larvae in the moderate paralysis stage. (D) Poisoned larvae in the deep paralysis stage. Chlorantraniliprole-treated larvae: (F) Poisoned larvae in the preliminary poisoning stage. (G) Poisoned larvae the moderate poisoning stage. (H) Poisoned larvae in the later poisoning stage.

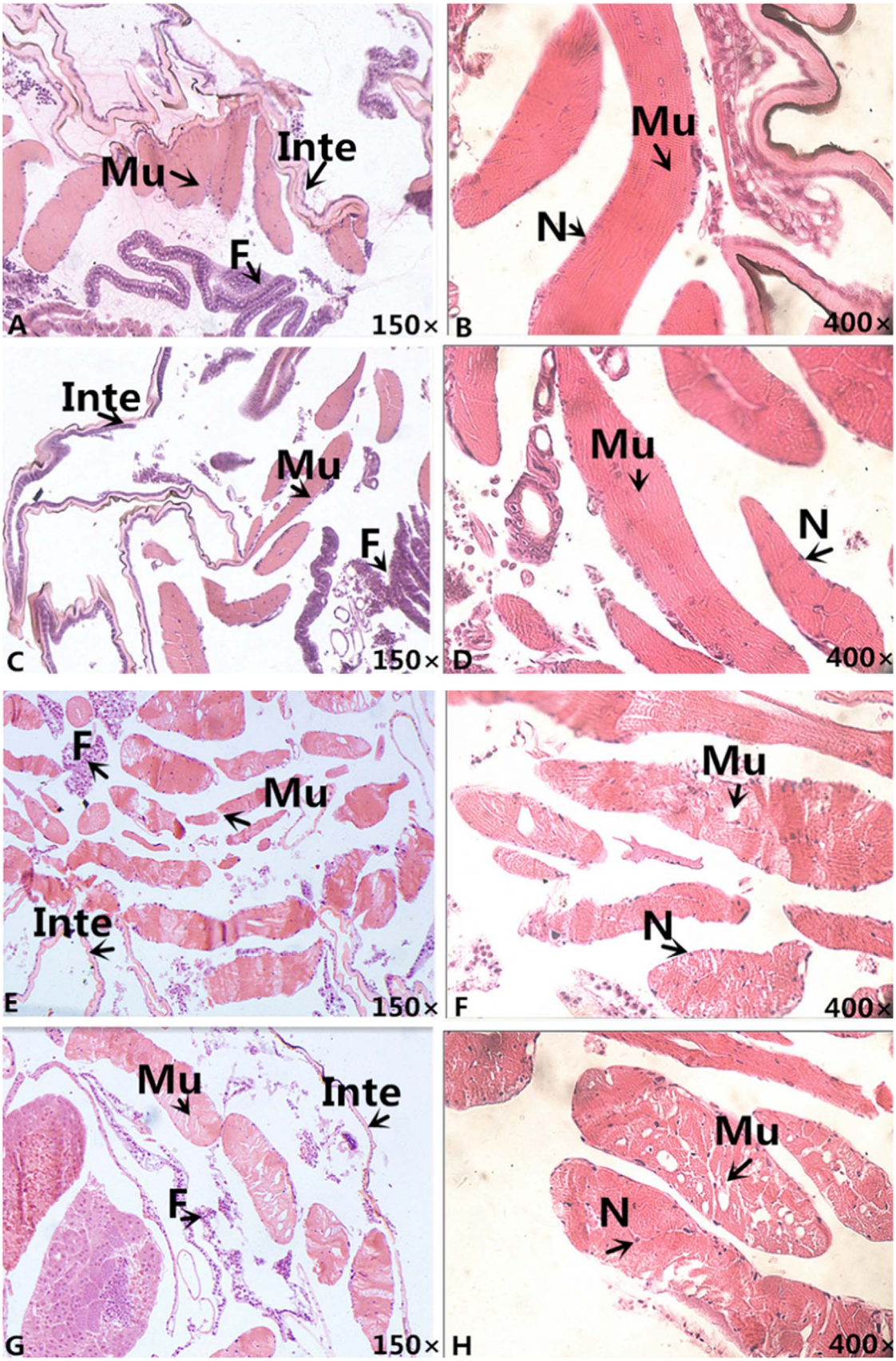

Fig. 3. Microstructural changes of the somatic muscle of *M. separata* larvae after ingestion of wilforgine. (A and B) Uniform thickness of the integuments was found and lots of fat bodies adhered to them; cell nuclei were intact, and muscle fibers were stained to a regular pattern in the control larvae. (A) 150× and (B) 400×. (C and D) Discontinuous integuments of the treated larvae were observed, and fat bodies were fewer. Intact cell nuclei could also be observed, and muscle fibers were stained in an irregular pattern in the weak paralysis stage. (C) 150× and (D) 400×. (E and F) More discontinuous integuments were observed and were partly disordered. Part of the fat bodies began to break up, and the muscle fibers were arranged more irregularly in the moderate paralysis stage. (E) 150× and (F) 400×. (G and H) The integuments were completely discontinuous and disordered, and fat bodies were extremely broken up. Cell nuclei were stained deeply, and muscle fibers were arranged irregularly in the deep paralysis stage. (G) 150× and (H) 400×. Mu, Muscle fiber; Inte, integument; F, Fat; N, Nucleus.

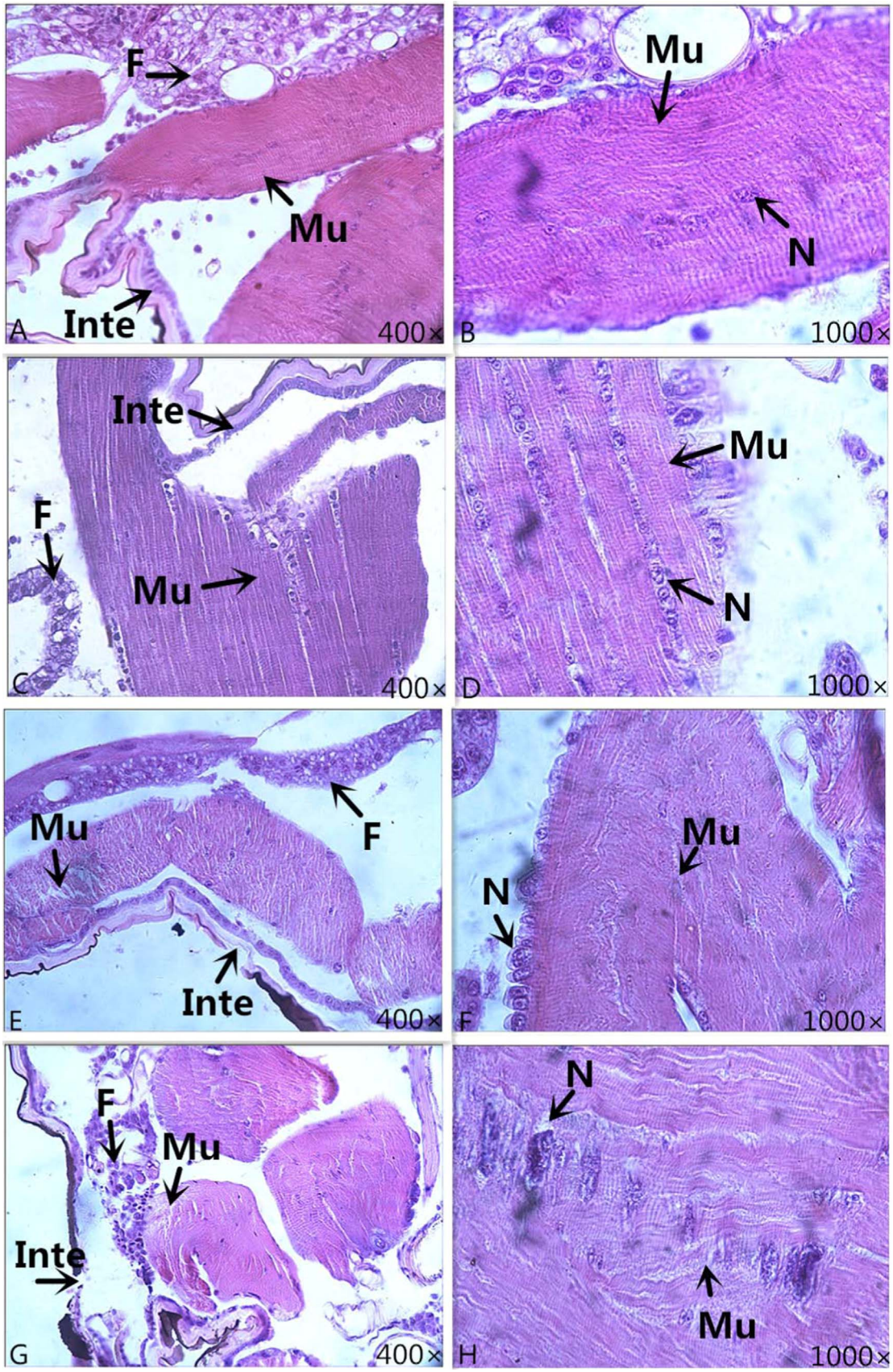

Fig. 4. Microstructural changes of the somatic muscle of *M. separata* larvae after ingestion of chlorantraniliprole. (A and B) The integuments had uniform thickness and fat bodies were adhering to them; intact cell nuclei and regular muscle fibers were observed in the control larvae. (A) 400× and (B) 1000×. (C and D) Integuments of different thickness and fewer fat bodies were observed. Cell nuclei were intact, and muscle fibers were irregular in the preliminary poisoning stage. (C) 400× and (D) 1000×. (E and F) Parts of integuments were disordered and the muscle fibers exhibited an irregular staining pattern at the moderate poisoning stage. (E) 400× and (F) 1000×. (G and H) The integuments were completely disordered, and fat bodies were very broken up. Irregularly arranged muscle fibers were also observed at the later poisoning stage. (G) 400× and (H) 1000×. Mu, Muscle fiber; Inte, integument; F, Fat; N, Nucleus.

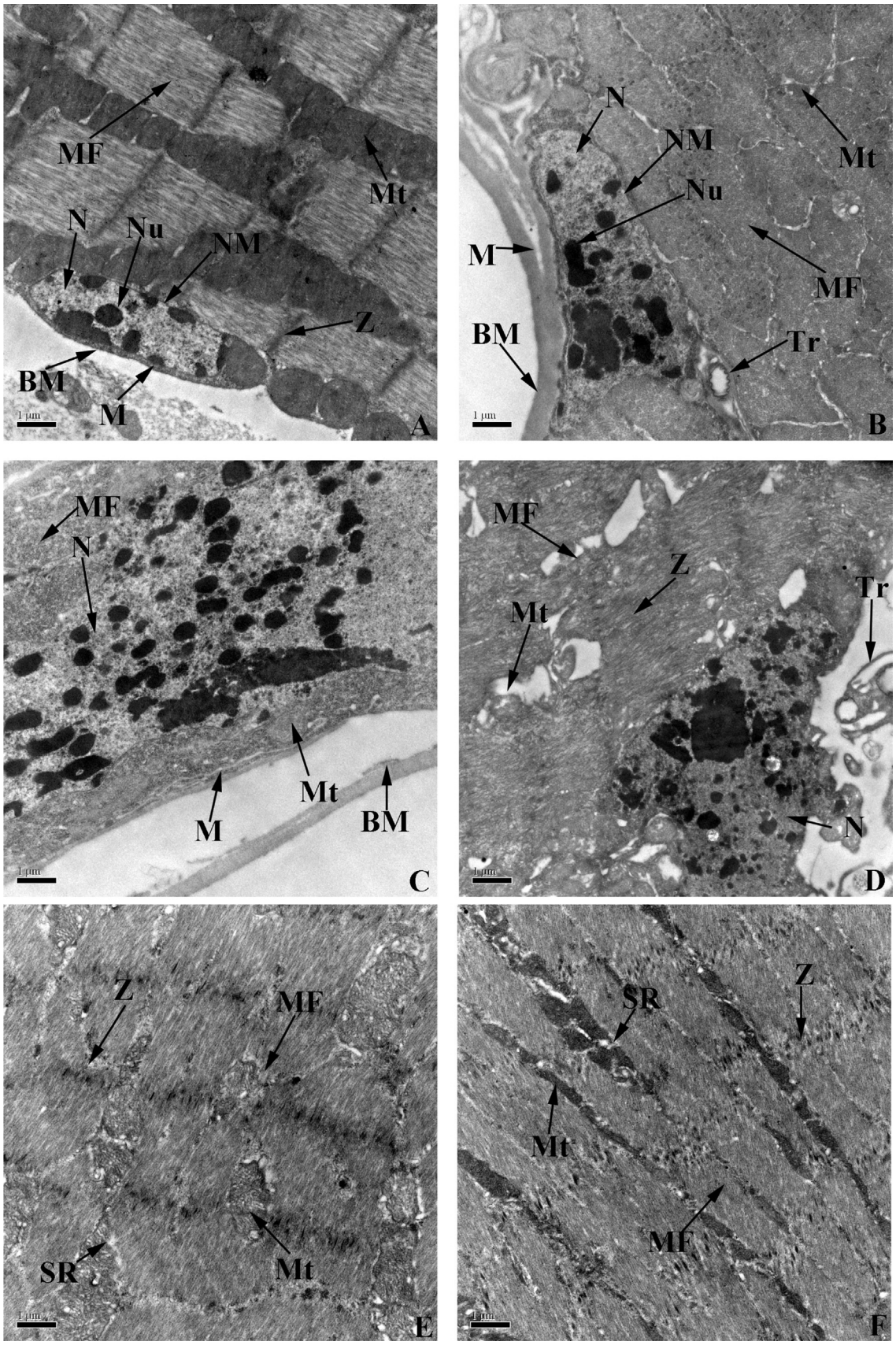
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Fig. 5. Ultrastructural changes in the somatic muscle of *M. separata* larvae after ingestion of wilforgine.(A, E and I) Control larvae: the structure of myocytes was intact; cell nuclei were complete with an intact double membrane, uniform nuclear material, and clear nucleolus. The Z-lines and myofibrils were regular; the SR was intact, tight and surrounded by myofibrils. Mitochondria complete with an intact double membrane were round or oval, and the crests of mitochondria were rich and full. (A and E) Bar, 1 μm; (I) Bar, 0.2 μm. (B, F and J) Wilforgine-treated larvae: in the weak paralysis stage, cell-nuclei membranes were partially damaged; a blurred nucleolus and concentrated nuclear material could be observed. The Z-lines were faint and myofibrils began to break up, and the SR started to expand. Swollen mitochondria and fractured cristae were observed. (B and F) Bar, 1 μm; (J) Bar, 0.2 μm. (C, G and K) In the moderate paralysis stage, the nuclear membranes were damaged; the nucleolus was absent and nuclear material was concentrated to one site. The Z-lines were fuzzy and myofibrils fractured, and the SR continued to expand. Mitochondria appeared swollen and vacuolated and the cristae were very fractured. (C and G) Bar, 1 μm; (K) Bar, 0.2 μm. (D, H and L) In the deep paralysis stage, the nuclear membranes disappeared completely, and nuclear material was concentrated to one site. The Z-lines were fuzzy and partially disappeared, myofibrils were very fractured, and the SR expanded deeply and was arranged erratically. Mitochondria were swollen and fractured, and cristae disappeared completely. (D and H) Bar, 1 μm; (L) Bar, 0.2 μm. BM, Basilar membrane; M, Muscular membrane; N, Nucleus; Nu, Nucleolus; MF, Myofibril; Mt, Mitochondria; Z, Z-line; SR, Sarcoplasmic reticulum; Tr, tracheole; G, Golgi; MS, Medullary sheath structure; CL, Crystalline inclusion.

extreme contraction of the body and the length of larvae shortened by one-half, and the larvae died after 2–3 days (Fig. 2H).

3.2. LM observations

3.2.1. Wilforgine-treated larvae

Observations of the somatic muscles of *M. separata* larvae treated with wilforgine are shown in Fig. 3. In the control group, uniform thickness of the integuments was found and lots of fat bodies adhered to them (Fig. 3A); cell nuclei were intact, and muscle fibers were stained to a regular pattern (Fig. 3B). In the initial weak-paralysis stage, discontinuous integuments of the treated larvae were observed, and fat bodies were fewer (Fig. 3C). Intact cell nuclei could also be observed, and muscle fibers were stained in an irregular pattern (Fig. 3D). In the moderate-paralysis stage, more discontinuous integuments were observed and were partly disordered. Part of the fat bodies began to break up, and the muscle fibers were arranged more irregularly (Fig. 3E, F). In the deep-paralysis stage, the integuments were completely discontinuous and disordered, and fat bodies were extremely broken up (Fig. 3G). Cell nuclei were stained deeply, and muscle fibers were arranged irregularly (Fig. 3H).

3.2.2. Chlorantraniliprole-treated larvae

The somatic muscle of *M. separata* larvae treated with chlorantraniliprole was observed under LM (Fig. 4). In the control group, the integuments had uniform thickness and fat bodies were adhering to them (Fig. 4A); intact cell nuclei and regular muscle fibers were observed (Fig. 4B). During the preliminary-poisoning stage, integuments of different thickness and fewer fat bodies were observed (Fig. 4C). Cell nuclei were intact, and muscle fibers were irregular (Fig. 4D). At the moderate-poisoning stage, parts of integuments were disordered and the muscle fibers exhibited an irregular staining pattern (Fig. 4E, F). At the late-poisoning stage, the integuments were completely disordered, and fat bodies were very broken up. Irregularly arranged muscle fibers were also observed (Fig. 4G, H).

3.3. TEM observations

3.3.1. Wilforgine-treated larvae

The ultrastructure of the somatic muscle of wilforgine-treated *M. separata* larvae was observed by TEM. In the control larvae, the structure of myocytes was intact. Cell nuclei were complete with an intact double membrane, uniform nuclear material, and clear nucleolus (Fig. 5A). The Z-lines and myofibrils were regular; the SR was intact, tight and surrounded by myofibrils (Fig. 5E). Mitochondria complete with an intact double membrane were round or oval, and the crests of mitochondria were rich and full (Fig. 5I). In the weak-paralysis stage of wilforgine-treated larvae, cell-nuclei membranes were partially damaged; a blurred nucleolus and concentrated nuclear material could be observed (Fig. 5B). The Z-lines were faint and myofibrils began to break up, and the SR started to expand (Fig. 5F). Swollen mitochondria and fractured cristae were observed (Fig. 5J). During the moderate-paralysis period, the nuclear membranes were damaged; the nucleolus was absent and nuclear material was concentrated to one site (Fig. 5C). The Z-lines were fuzzy and myofibrils fractured, and the SR continued to expand (Fig. 5G). Mitochondria appeared swollen and vacuolated and the cristae were very fractured (Fig. 5K). In the deep-paralysis stage, the nuclear membranes disappeared completely, and nuclear material was concentrated to one site (Fig. 5D). The Z-lines were fuzzy and partially disappeared, myofibrils were very fractured, and the SR expanded deeply and was arranged erratically (Fig. 5H). Mitochondria were swollen and fractured, and cristae disappeared completely (Fig. 5L).

3.3.2. Chlorantraniliprole-treated larvae

TEM of thin sections of the somatic muscle of chlorantraniliprole-treated *M. separata* larvae are shown in Fig. 6. In the control larvae, myocyte structure was intact; the double membrane of cell nuclei could be observed; nuclear material was uniform and nucleoli were very clear (Fig. 6A). The Z-lines had a regular shape, neatly arranged myofibrils could be observed, and the SR was intact, tight and surrounded by myofibrils (Fig. 6E). Round or oval mitochondria were observed, mitochondria with a double membrane were intact, and mitochondria crests were full and rich (Fig. 6I). During the preliminary-poisoning stage after chlorantraniliprole treatment, the myocyte structure began to change. That is, the nuclear membrane was partially dissolved, the nuclear material was concentrated to one site, and the nucleoli appeared swollen (Fig. 6B). The Z-lines were intermittent, part of the myofibrils began to fracture, and dilated SR could be observed (Fig. 6F). The mitochondria were irregularly swollen and cristae were partly fractured (Fig. 6J). At the moderate-poisoning stage, the double membrane of cell nuclei was damaged, the nucleolus disappeared, and nuclear material remained concentrated to one site (Fig. 6C). The Z-lines were unclear and myofibrils fractured continuously; and the SR ruptured irregularly (Fig. 6G). The cristae of the mitochondria were very fractured and blank areas appeared (Fig. 6K). The structure of muscle cells was completely destroyed at the late-poisoning stage. All the nuclear membranes disappeared, and nuclear material was concentrated to one site (Fig. 6D). The Z-lines nearly completely disappeared, myofibrils were disorderly arranged and the SR exhibited irregular

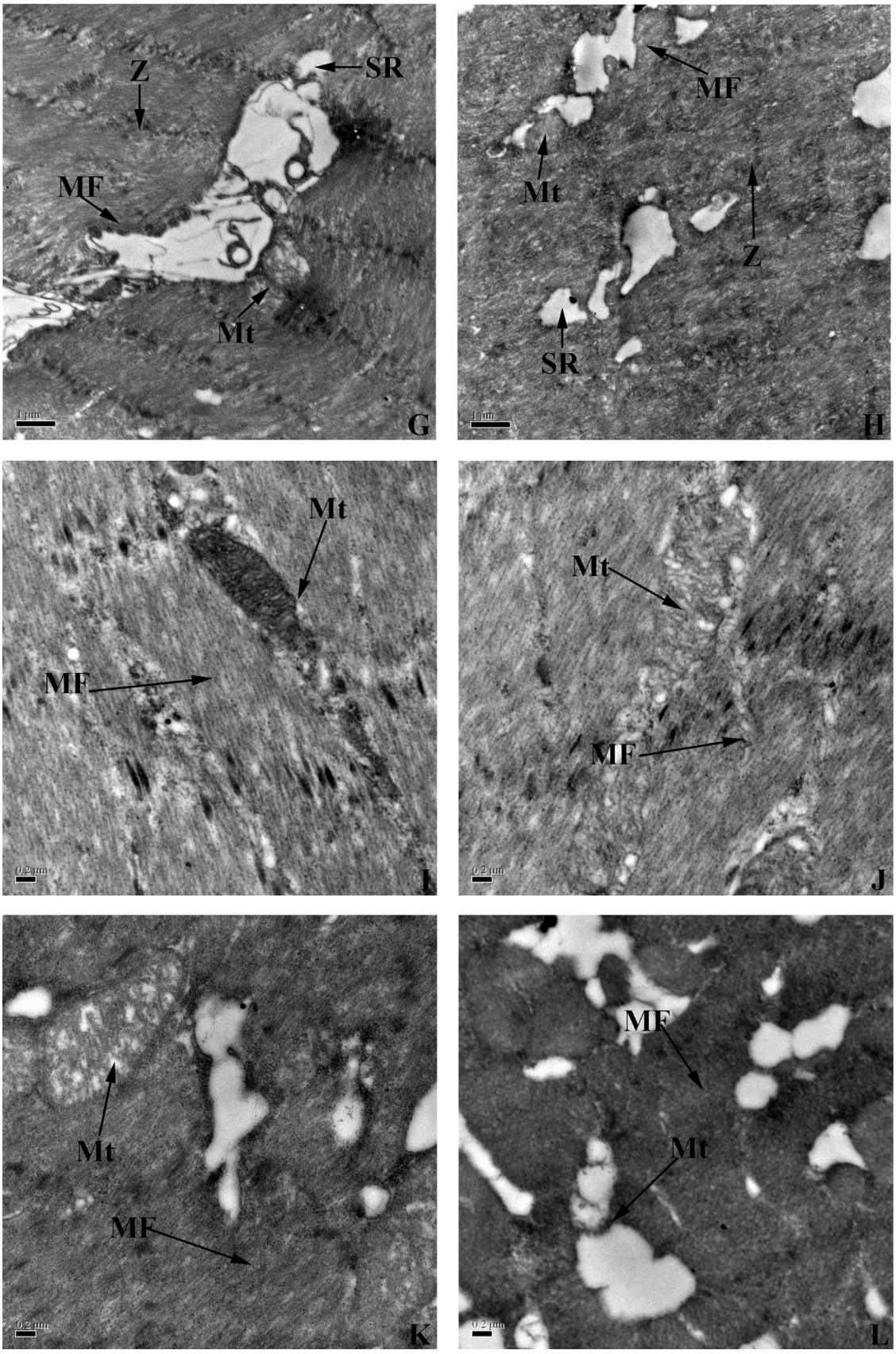

Fig. 5. (*continued*)

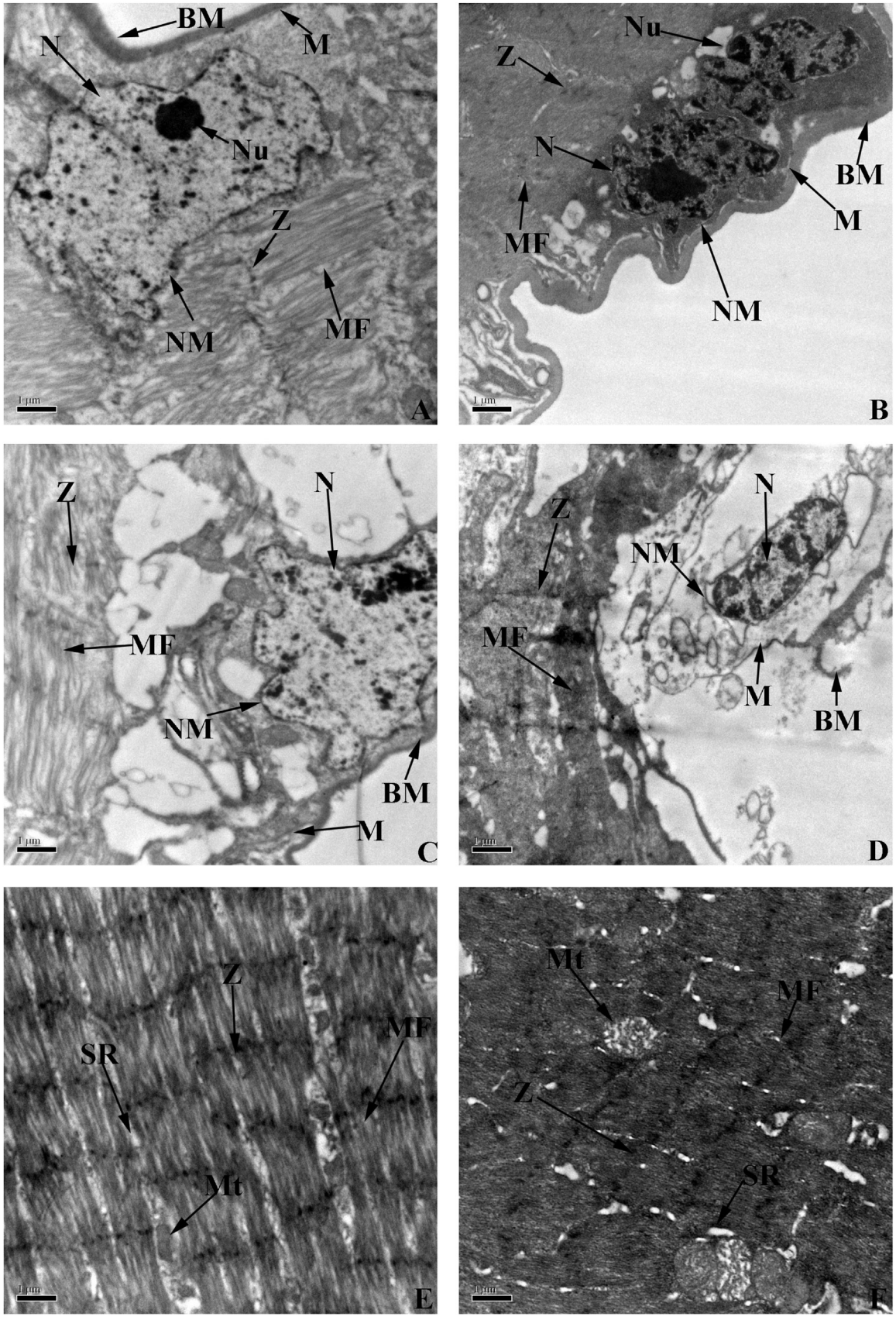
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Fig. 6. Ultrastructural changes of the thoracic dorsal-longitudinal muscle of *M. separata* adults after ingestion of chlorantraniliprole. (A, E and I) Control larvae: myocyte structure was intact; the double membrane of cell nuclei could be observed; nuclear material was uniform and nucleoli were very clear. The Z-lines had a regular shape, neatly arranged myofibrils could be observed, and the SR was intact, tight and surrounded by myofibrils. Round or oval mitochondria were observed, mitochondria with a double membrane were intact, and mitochondria crests were full and rich. (A and E) Bar, 1 μm; (I) Bar, 0.2 μm. (B, F and J) Chlorantraniliprole-treated larvae: in the preliminary poisoning stage, the myocyte structure began to change. That is, the nuclear membrane was partially dissolved, the nuclear material was concentrated to one site, and the nucleoli appeared swollen. The Z-lines were intermittent, part of the myofibrils began to fracture, and dilated SR could be observed. The mitochondria were irregularly swollen and cristae were partly fractured. (B and F) Bar, 1 μm; (J) Bar, 0.2 μm. (C, G and K) At the moderate poisoning stage, the double membrane of cell nuclei was damaged, the nucleolus disappeared, and nuclear material remained concentrated to one site. The Z-lines were unclear and myofibrils fractured continuously; and the SR ruptured irregularly. The cristae of the mitochondria were very fractured and blank areas appeared. (C and G) Bar, 1 μm; (K) Bar, 0.2 μm. (D, H and L) At the later poisoning stage, all the nuclear membranes disappeared, and nuclear material was concentrated to one site. The Z-lines nearly completely disappeared, myofibrils were disorderly arranged and the SR exhibited irregular rupturing. Mitochondria were fractured and showed blank areas, and the cristae of mitochondria had disappeared completely. (D and H) Bar, 1 μm; (L) Bar, 0.2 μm. BM, Basilar membrane; M, Muscular membrane; NM, nuclear membrane; N, Nucleus; Nu, Nucleolus; MF, Myofibril; Mt, Mitochondria; Z, Z-line; SR, Sarcoplasmic reticulum.

rupturing (Fig. 6H). Mitochondria were fractured and showed blank areas, and the cristae of mitochondria had disappeared completely (Fig. 6K).

4. Discussion

The site of action of wilforgine may be in insect muscle tissue. Several studies have indicated that the poisoning symptoms caused by insecticides are based on paralysis of the nervous/muscular systems of insects. For example, *Periplaneta americana* L. is paralyzed if it is treated with abamectin (allosteric modulator of glutamate-gated chloride channels) (Corbitt et al., 1992; Wolstenholme and Rogers, 2005) and the main poisoning symptom of cartap (blocker of nicotinic acetylcholine receptor channels) in insects is paralysis (Araujo et al., 2015; Lee et al., 2004). In addition, the contraction paralysis caused by diamide insecticides (modulators of ryanodine receptors) in insects is obvious (Ebbinghaus-Kintscher et al., 2006; Tohnishi et al., 2005) and ryanodine (modulator of ryanodine receptors) can cause paralysis in insects and animals (Rogers et al., 1948; Sutko et al., 1997). Also, celangulin I (modulator of a Ca^{2+} signaling pathway) can lead to paralysis in *M. separata* larvae (Li et al., 2016; Wu et al., 2005). In the present study, the typical symptom of wilforgine in *M. separata* larvae was paralysis, and further study showed that the microstructure of *M. separata* muscle cells could be damaged by wilforgine. Simultaneously, endomembranes and plasma membranes were disrupted. Thus, the muscular system may be the site of action of wilforgine.

The molecular target of wilforgine may be different from that of chlorantraniliprole. In general, the poisoning symptoms induced by insecticide are associated closely with its insecticidal mechanism (Tomizawa and Casida, 2005). We found that wilforgine and chlorantraniliprole could cause feeding cessation and paralysis. However, the paralysis symptoms induced by these agents were different: wilforgine could lead to flaccid paralysis and chlorantraniliprole brought about contraction paralysis. LM observations also showed different histopathologic changes. The muscle tissue of *M. separata* larvae became loose after wilforgine treatment, but constricted muscle tissue was observed after chlorantraniliprole treatment. In consideration of the different paralysis symptoms and muscle-tissue lesions, we propose that the molecular MoA of wilforgine differs from that of chlorantraniliprole. The latter modulates ryanodine receptors, which leads to an increase of intracellular Ca^{2+} concentration in muscle cells (Cordova et al., 2006; Lahm et al., 2009). The botanical pesticide celangulin I can affect ryanodine receptors and inositol 1,4,5-trisphosphate (IP_3) expression, resulting in the disruption of intracellular Ca^{2+} homeostasis, and flaccid paralysis of insects (Li et al., 2016). Based on the information stated above, it can be conjectured that wilforgine may disrupt Ca^{2+} homeostasis and reduce intracellular Ca^{2+} concentrations, leading to flaccid paralysis of *M. separata* larvae. Modulation of a Ca^{2+} signaling pathway needs the participation of receptors or channels, such as voltage-gated calcium channels, ryanodine receptors, IP_3, calcium pumps, and calmodulin. Thus, the molecular target of wilforgine may be one of these receptors or channels.

5. Conclusions

Wilforgine can induce microstructural and ultrastructural changes in the muscles of *M. separata* larvae; the sites of action are proposed to be calcium receptors or channels in the muscular system. Once the molecular target of wilforgine is elucidated, this may give an indication as to why wilforgine is highly selective for insect pests. Based on the ecological safety and high toxicity to pests, it is necessary to further elucidate the molecular insecticidal mechanism of wilforgine.

Conflict of interest

The authors have no conflicts of interest to declare.

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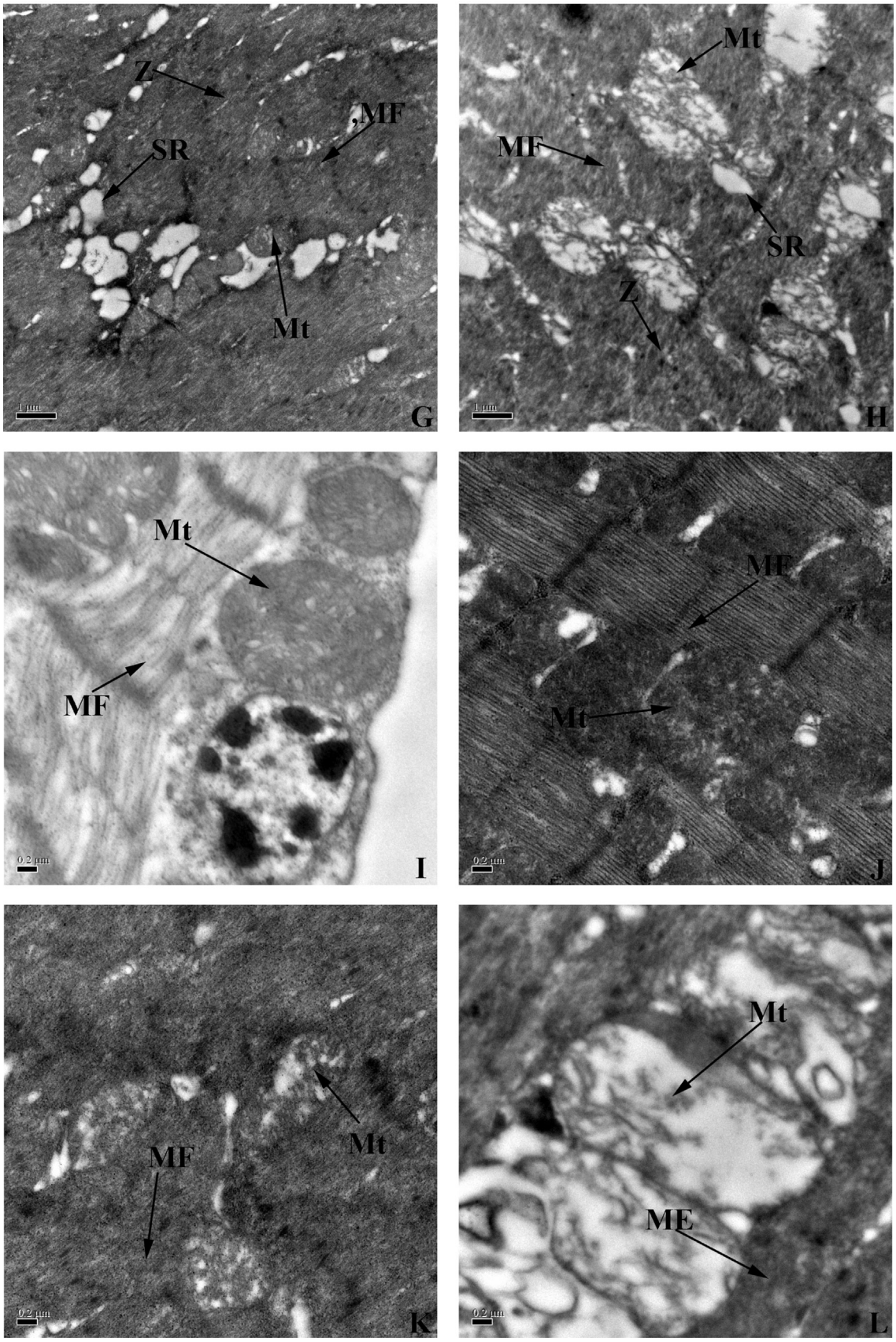

Fig. 6. (*continued*)

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