Suppression of Trehalase Activity by Validamycin Induces Mortality and Developmental Delays in *Sitophilus zeamais* (Coleoptera: Curculionidae)[1]

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J. Entomol. Sci. 59(3): 311–322 (July 2024)
DOI: 10.18474/JES23-62

Abstract The maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), is a major pest of stored grains that feeds on and develops inside the grain. The trehalose analog validamycin has a strong inhibitory effect on insect trehalase. Studies have found that validamycin affects several insects; however, no information is available on its inhibitory and physiological effects on these insects. In this study, validamycin's inhibitory effect on trehalase was examined by incorporating it into artificial seeds and feeding them to *S. zeamais*. Trehalase activity was recorded throughout the developmental stages of the exposed weevils and was highest in the larval followed by adult, pupal, and egg stages. We found that feeding adult *S. zeamais* artificial seeds containing validamycin resulted in a significant reduction of trehalase activity in both males and females, although the primary source of trehalase was in the intestinal tract rather than in the reproductive organs. The validamycin treatment also had a concentration-dependent lethal effect, resulting in approximately 90% mortality, with females being more susceptible than males. In addition, the validamycin treatment caused a significant reduction in the number of first-generation progeny and *S. zeamais* demonstrated delayed development in a concentration-dependent manner. These data show that validamycin influences trehalase enzyme activity, reproductive success, and development of *S. zeamais*. The results obtained from this research will be valuable tools for designing a control strategy.

Key Words maize weevil, validamycin, trehalase, artificial seed, trehalase inhibitor

Trehalose is the major circulating sugar in insects and consists of two glucose molecules linked by a 1,1-glycosidic bond; thus, it can be stored at high concentrations in the hemolymph (Becker et al. 1996). Trehalose is hydrolyzed by the glycolytic enzyme trehalase (EC3.2.1.28) and is found in many types of organisms (Thompson 2003). In insects, trehalase activity is crucial for energy supply, metamorphosis, chitin synthesis, and flight (Wegener et al. 2010, Chen et al. 2010, Luo et al. 2022) and changes in trehalase activity are directly linked to insect growth and development (Terra and Ferreira 1994, Silva et al. 2004, Tatun et al. 2014). If there is any interference with trehalose metabolism, it can cause physiological impairments in an insect's growth, development, and reproduction. Many studies

[1]Received 7 September 2023; accepted for publication 10 October 2023.
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have mentioned that suppression of trehalase in insects induces abnormal morphology as well as abnormal biology and biochemistry (Wegener et al. 2010); thus because trehalose analogs have the ability to inhibit trehalase, they have potential as bioinsecticides, fungicides, or antibiotics. As a result, there has been interest in the development of trehalase inhibitors as insecticidal agents (García and Argüelles 2021).

Validamycin is synthesized by the bacterium *Streptomyces hygroscopicus* Jensen and has been used to control sheath blight disease in rice (Asano 2003). The structure of validamycin is similar to that of trehalose; thus, it is a very strong competitive inhibitor of trehalase. Validamycin has been reported to suppress the trehalase enzymatic activity in various organisms, including insects from different orders: Lepidoptera (*Mamestra brassicae* (L.) and *Spodoptera frugiperda* (J.E. Smith)) (Kono et al. 1994, Luo et al. 2022), Diptera (*Musca domestica* L.) (Takahashi et al. 1995), Orthoptera (*Periplaneta fuliginosa* (Serville) and *Blattella germanica* (L.)) (Kono et al. 1999), Isoptera (*Odontotermes feae* Wasmann) (Tatun et al. 2014), and Coleoptera (*Tribolium castaneum* (Herbst)) (Tatun et al. 2016). Several studies have noted that validamycin has insecticidal properties and has been considered a control agent for insect pests; however, there are no reports of validamycin inhibiting the trehalase activity in maize weevils, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae).

The maize weevil is a pest that damages stored products, such as maize and other cereals. Its larvae feed and grow inside the maize kernels, resulting in a reduction in the weight of the stored grain (Noomhorm et al. 2009). Methyl bromide and phosphine are commonly used worldwide as effective methods to control infestations of storage pests; however, these fumigants have resulted in serious environmental damage and are a hazard to human health. Thus, it is necessary to find new environmentally friendly pest control methods.

The aim of this study was to investigate the impact of validamycin on the maize weevil. To achieve this objective, we examined the changes in trehalase activity and mortality rate over 7 d in *S. zeamais* reared on artificial seeds mixed with various concentrations of validamycin. We also analyzed how trehalase activity responded to validamycin in male versus female *S. zeamais*. Furthermore, we examined the trehalase activity in all developmental stages of *S. zeamais* that were reared on an artificial diet. Last, the morphological changes in first-generation (F1) progeny developed from validamycin-treated artificial seeds were recorded to evaluate the impact of validamycin on insect development.

Materials and Methods

Insects. Maize weevils were obtained from the Postharvest and Processing Product Research and Development Office, Department of Agriculture, Thailand. The insect cultures were maintained on heat-sterilized brown rice grains in a wide-mouthed glass bottle. The bottle was enclosed with a double layer of cheesecloth held in position by rubber bands and stored in an incubator at 30 ± 1°C at 70 ± 5% relative humidity.

Preparation of artificial seeds. Because maize weevils naturally feed and develop in corn kernels, we prepared artificial seeds as their diet. A validamycin-treated diet was prepared by mixing validamycin stock (30 μg/ml) with 100 g of rice

flour plus 5% of yeast to yield 0.0625-, 0.125-, 0.25-, 0.5-, 1-, 2-, and 3-μg/ml concentrations of validamycin. We then placed this mixed diet into artificial seed molds by using a method similar to that described by Holmes et al. (2020), with a minor modification to create round artificial seeds that were approximately 0.25 mg in weight and similar in size to corn kernels. Finally, we dried these artificial seeds at 37°C for 48 h in an incubator.

Validamycin treatment. Artificial seeds ($n = 70$), including control and validamycin mixed diets at concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 3 μg/ml, were placed individually in a wide-mouthed glass bottle. Adult *S. zeamais* (10 females and 10 males) were released into each bottle and left for 3 d to feed and lay eggs on the artificial seeds. After 3 d, they were removed from the rearing bottle. The number of F1 progeny that developed from each treatment was recorded. In addition, both the artificial seeds of the control and validamycin-treated groups were dissected under a stereomicroscope to observe the developmental changes of the weevils at days 19 and 22 after exposure to validamycin and a photo was digitally captured. The experiment was replicated three times.

In addition, male ($n = 10$) and female ($n = 10$) adult *S. zeamais* were placed in a glass bottle containing artificial seeds mixed with different concentrations of validamycin in a separate bottle for 7 d. Mortality rate was then determined. Male ($n = 10$) and female ($n = 10$) adult *S. zeamais* were placed separately in the glass bottle containing artificial seeds mixed with 0.5 μg/ml validamycin for 7 d. A sample was collected every day to measure the changes in trehalase activity after validamycin ingestion. The experiment was replicated three times.

Preparation of *S. zeamais* trehalase. To examine the developmental changes in trehalase activity, a specimen of each developmental stage (30 eggs, 5 young larvae, 5 late-stage larvae, 5 prepupae, 5 pupae, and 3 male and female adults) was collected and placed in a microcentrifuge tube, with three replications for each sample. A sample was homogenized using a plastic pestle in 200 μl of 20 mM phosphate buffer (PB; pH 6.0). Next, the tube was placed in the ultrasonic bath for 10 s three times to maximize cell lysis. The homogenized samples were centrifuged at 14,000 rpm at 4°C for 10 min, and the insect extract was kept at −20°C for the trehalase activity assay. In addition, the *S. zeamais* adults were dissected to collect their gut, testis, and ovary and subjected to a trehalase assay. The experiment was replicated three times.

Trehalase activity assessment. Trehalase activity was assessed by detecting glucose generation from trehalose hydrolysis. The reaction mixture consisted of 50 μl of the insect extract, 130 μl of 40 mM trehalose (Sigma, St. Louis, MO), and 70 μl of 20 mM PB (pH 6.0) in the microcentrifuge tube. The reaction tube was incubated in a water bath at 37°C for 60 min while shaking. To stop the reaction after incubation, the mixture was boiled at 100°C for 5 min and then allowed to cool on ice for 5 min. Finally, the sample was centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was transferred to a new tube to examine the quantity of glucose. The reaction mixture, including 500 μl of 20 mM PB, 250 μl of 630 mM triethanolamine buffer (pH 7.0; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 125 μl of 2.0 mM nicotinamide adenine dinucleotide phosphate (Sigma), and 125 μl of ATP (Sigma), was prepared in a 1.5-ml centrifuge tube. Next, a sample (100 μl) was added to the reaction mixture, incubated at 25°C for 60 min, and transferred to a 1.5-ml plastic cuvette. The absorbance at 340 nm was measured

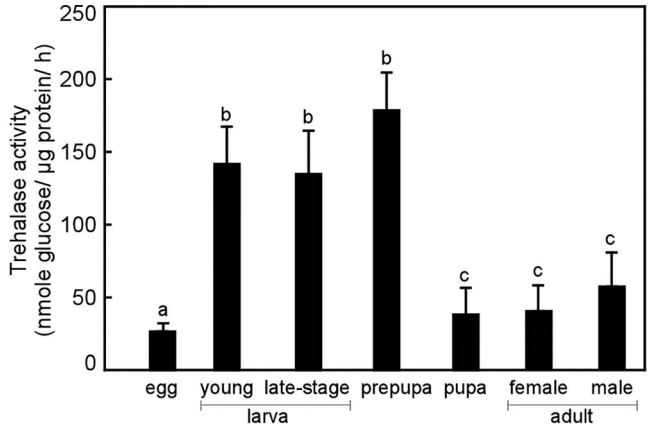

Fig. 1. Developmental changes in *Sitophilus zeamais* trehalase activity reared in artificial seeds. Trehalase activity is expressed in nanomoles of glucose per microgram of protein per hour. Error bars indicate SD, and the values labeled with different letters are significantly different ($P < 0.05$).

using a ultraviolet-visible spectrophotometer (BioMate^{TM} 3S, Thermo Fisher Scientific, Waltham, MA), and the concentration of generated glucose was calculated using the glucose (Sigma) standard curve. The protein amount in each enzyme sample solution was measured using the protein dye-binding method (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard.

Observation of developmental changes after the validamycin treatment. After the *S. zeamais* adults were released into the artificial seed mixed with different concentrations of validamycin, the infested seeds were kept until the adult weevils emerged. On days 19 and 22 posttreatment, the infested seeds were dissected under a stereomicroscope (Olympus Corporation, Tokyo, Japan) to observe the development of the weevils. The larvae, pupae, and adult weevils were photographed using a stereomicroscope (Olympus Corporation) fitted with a digital camera. The photos were documented using Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA) and imaging software cellSens 1.8 (Olympus Corporation).

Statistical analysis. One-way analysis of variance and a least-significance difference multiple range test were used for all statistical analyses (SPSS 11.5). The significance level was set to 0.05 ($P < 0.05$).

Results

Developmental change of trehalase activity in *S. zeamais*. The trehalase activity in the egg stage was the lowest level (27.41 $\pm$ 5.07 [mean $\pm$ SD] nmol glucose/µg protein/h) recorded compared with that of the other stages (Fig. 1). It increased five times during the young-stage larvae (143.14 $\pm$ 25.07 nmol glucose/µg protein/h) and remained high during the late-stage larval and prepupae stages (136.03 $\pm$ 82.24 and 175.30 $\pm$ 53.64 nmol glucose/µg protein/h, respectively).

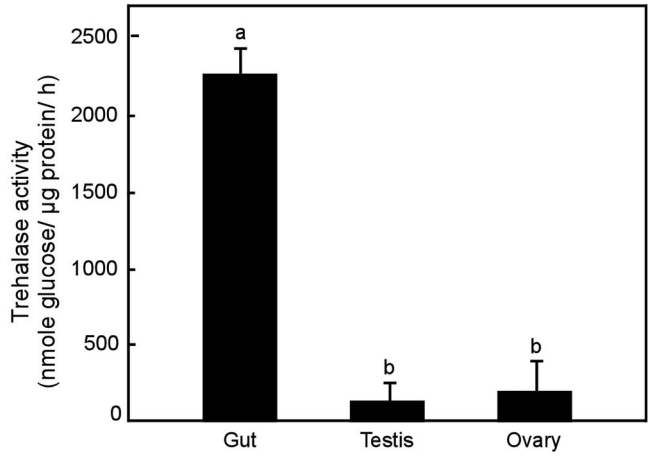

Fig. 2. Distribution of trehalase activity in gut, testis, and ovary of *Sitophilus zeamais*. Trehalase activity is expressed in nanomoles of glucose per microgram of protein per hour. Error bars indicate SD, and the values labeled with different letters are significantly different ($P < 0.05$).

The activity decreased significantly during the pupal stage (38.79 ± 11.38 nmol glucose/µg protein/h). In addition, the adult stage exhibited a similar level of trehalase activity in both males and females. The highest level of trehalase activity in *S. zeamais* was recorded in the gut. Trehalase activity was also detected in the reproductive organs; however, the testis and ovary exhibited approximately 11-fold lower levels than in the gut (Fig. 2).

Number of F1 progeny. The results showed that the number of F1 progeny was similar among the control group and the groups treated with 0.0625–0.5 mg/ml validamycin (Fig. 3). Interestingly, the number of F1 progeny decreased significantly when weevils were treated with 1.0 µg/ml and had the lowest number in the 2- to 3-µg/ml validamycin-treated group.

Developmental changes in F1 progeny. When the artificial seeds in the control group were dissected after 19 d (Fig. 4A), it was observed that the weevils had developed to the pupal stage and had a creamy color. The body length and width was recorded as 3.78 ± 0.27 and 1.74 ± 0.15 mm, respectively. In the artificial seed groups treated with 0.0625–0.5-mg/ml validamycin (Fig. 4B–E), the weevils were observed to be at the larval stage and had a creamy color. The body size of the larvae developed in 0.0625- and 0.125-µg/ml validamycin-treated seed groups was similar, with a length of 2.32 ± 0.21 mm and a width of 1.78 ± 0.11 mm (Fig. 5B, C). However, the body size of the larvae developed in 0.25- and 0.5-µg/ml validamycin-treated seed groups decreased significantly to 1.75 ± 0.08 and 1.48 ± 0.15 mm in length and 1.34 ± 0.04 and 1.28 ± 0.10 mm in width, respectively (Fig. 4D, E). Interestingly, when the artificial seeds were treated with higher concentrations of validamycin (1–3 µg/ml), very small and dead larvae were observed (Fig. 4F–H).

After the infested artificial seeds were dissected on day 22 of the experiment, the adult weevils were observed in the control group to have a dark brown color

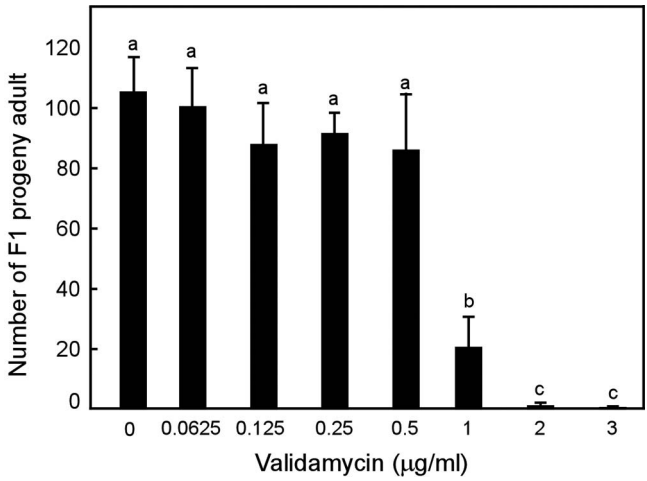

Fig. 3. Number of first-generation (F1) progeny adult *Sitophilus zeamais* resulted from validamycin-treated adults at various concentrations. Error bars indicate SD, and the values labeled with different letters are significantly different ($P < 0.05$).

and their body size was measured as 4.51 ± 0.37 mm in length and 1.26 ± 0.15 mm in width (Fig. 5A). By contrast, pupae of similar size were found in the artificial seed groups treated with 0.06525 μg/ml validamycin (Fig. 5B–D). At the same time, small larvae were observed in the artificial seed group treated with 0.5 μg/ml validamycin. Interestingly, in the artificial seed group treated with higher concentrations of validamycin (1–3 μg/ml), dead larvae were found (Fig. 5F–H).

Mortality effect of validamycin on *S. zeamais*. After the adult *S. zeamais* ($n = 20$) were reared in validamycin-treated artificial seeds at various concentrations, the results showed that there was no mortality recorded in the control group. Mortality was recorded at an exceptionally low level in the groups treated with 0.0625–0.5-μg/ml validamycin. In addition, weevils reared on 1-, 2-, and 3-μg/ml validamycin-treated seeds had high mortality rates of 15, 19, and 20, respectively (Fig. 6A). Furthermore, when male and female adult weevils were treated with 0.5 μg/ml validamycin in a separate bottle, the mortality rate of the female weevils was 39.13%, a value that was higher than the mortality rate of the male weevils (13.46%; Fig. 6B).

Response of trehalase activity in male and female weevils to validamycin. After the adult male and female maize weevils were fed with validamycin (0.5 μg/ml) presented in the artificial seeds for 7 d, the level of trehalase activity was measured each day. The results showed that male maize weevils in the control group (day 0) had the highest trehalase activity (142.61 ± 11.38 nmol glucose/μg protein/h; Fig. 7A). Compared with the control group, the trehalase activity in the validamycin-treated groups decreased significantly to approximately 2.8 times lower on the first day post-treatment and remained at a low level until day 6 (51.01 ± 13.81, 36.38 ± 10.31, 62.85 ± 21.22, 45.44 ± 10.34, 35.48 ± 9.32, and 40.00 ± 11.25 nmol glucose/μg protein/h). However, the trehalase activity increased again on day 7 of the experiment

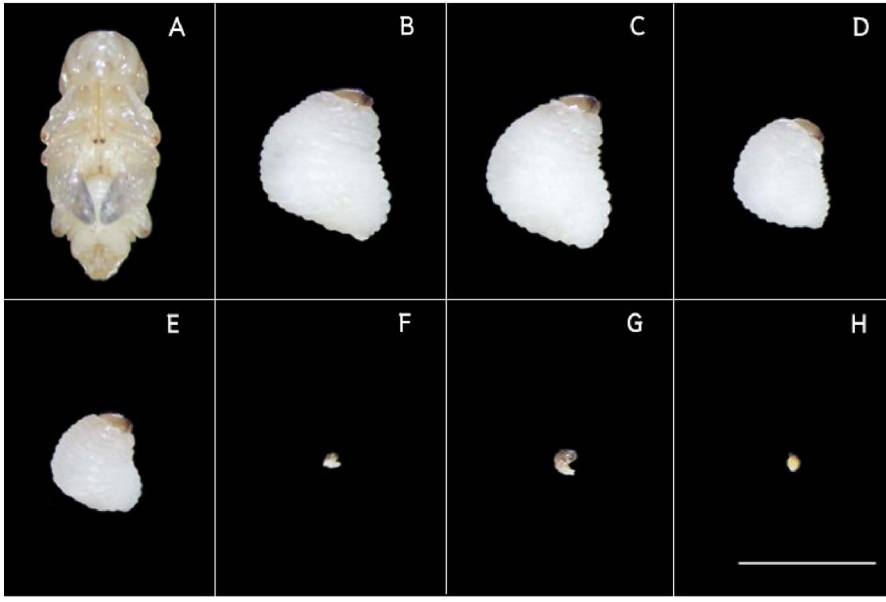

Fig. 4. Development of *Sitophilus zeamais* grown inside artificial seeds mixed with various concentrations of validamycin at 19 d of experiment. (A) Control pupa exhibited a creamy color with normal morphology. (B–C) Larvae had a creamy color and normal morphology when grown inside artificial seeds mixed with 0.0625 and 0.125 μg/ml validamycin. (D–E) Small larvae were observed in artificial seeds treated with 0.25 and 0.5 μg/ml validamycin, and (F–H) dried larvae were observed in artificial seeds treated with 1–3 μg/ml validamycin. Scale bar, 1 mm ($n = 30$).

to approximately two thirds of the activity observed in the control groups (91.26 ± 32.12 nmol glucose/μg protein/h).

Female weevils in the control group exhibited a little lower trehalase than male weevils (115.24 ± 55.32 nmol glucose/μg protein/h; Fig.7B). Similarly, trehalase activity in females in the validamycin-treated group on day 1 posttreatment started to decrease (45.82 ± 10.31 nmol glucose/μg protein/h), with a 1.5-fold lower level than that of the control group. The enzyme activity remained at a low level until the end of the experiment. In contrast with the male weevils, trehalase activity in female weevils on day 7 did not exhibit an increase; instead, it had a similar level compared with levels on days 1–6 posttreatment in which the trehalase activity ranged between 30.96 and 44.57 nmol glucose/μg protein/h.

Discussion

Because the development of *S. zeamais* occurs inside grain kernels, artificial seeds were used in this study to collect weevils at all developmental stages and investigate the changes in the enzymatic activity of trehalase. The lowest activity

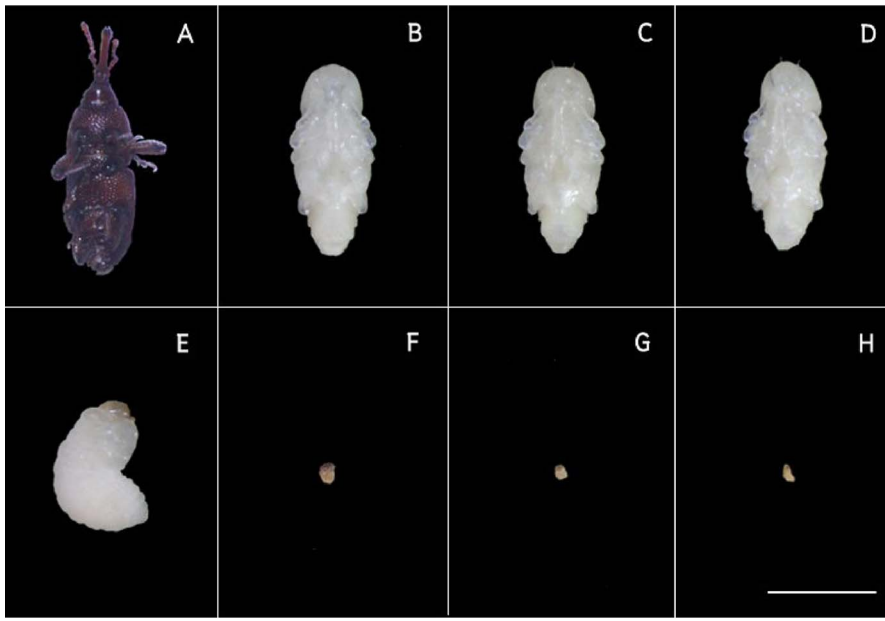

Fig. 5. Development of *Sitophilus zeamais* grown inside artificial seeds mixed with various concentrations of validamycin at 22 d of experiment. (A) Control adults exhibited a dark brown cuticle. (B–D) Pupae had a creamy color and normal morphology when grown inside artificial seeds mixed with 0.0625, 0.125, and 0.25 μg/ml validamycin. (E) larvae were observed in artificial seeds treated with 0.5 μg/ml validamycin, and (F–H) dried larvae were observed in artificial seeds treated with 1–3 μg/ml validamycin. Scale bar, 1 mm ($n = 30$).

was recorded in the eggs. Activity gradually increased during the larval stage, followed by a decrease in trehalase activity in the pupal and adult stages, which is consistent with that of other insect species studied. For example, the lowest trehalase activity was recorded in the eggs of *T. castaneum* and *Bombyx mori* (L.) (Katagiri et al. 1998, Tatun et al. 2014), whereas the highest trehalase activity was throughout the larval stages and related to insect feeding behavior. The insect gut has so far been reported as a major site of trehalase because trehalase has both digestive and physiological functions in insects (Gomez et al. 2013). Similarly, trehalase activity was at its highest level in the gut of *S. zeamais*. In addition, the reproductive organs of these weevils exhibit enzymatic activity of trehalase (Santos et al. 2012, Ma et al. 2015). The effects of validamycin have been investigated previously in many insects (Tang et al. 2017, Adhav et al. 2018, Marten et al. 2020, Yu et al. 2020); however, it is challenging to administer and observe validamycin's effect(s) on insects, including the maize weevil, that grow inside grains and kernels. In this study, we used artificial seeds as a mechanism to deliver the trehalase inhibitor validamycin to the larvae of *S. zeamais* because it has been confirmed that incorporating the compound of interest into artificial seeds is an

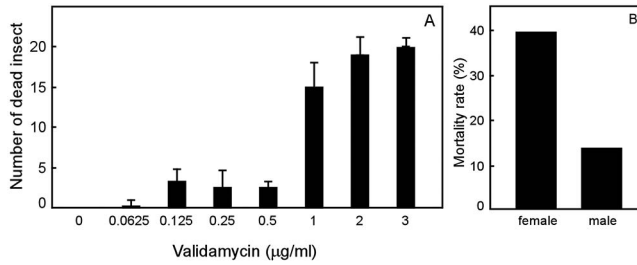

Fig. 6. Lethal effect of validamycin on *Sitophilus zeamais*. (A) Adults fed 0.0625, 0.125, 0.25, 0.5, 1, 2, and 3 μg/ml validamycin mixed with artificial seeds. (B) Mortality rate of males and females treated with 0.5 μg/ml validamycin present in artificial seeds.

effective method in assessing the bioactivity of bioactive agents against insects that grow and develop inside kernels (Hudaib et al. 2013, Holmes et al. 2020). Furthermore, we demonstrated that adult male and female *S. zeamais* responded to validamycin differently. The suppression of trehalase in females started on day 1 posttreatment and continued for 7 d of the experiment. By contrast, trehalase activity in male *S. zeamais* tended to increase to approximately two thirds of the control on day 7 of the experiment, suggesting that male *S. zeamais* recovered from the validamycin treatment earlier than females. Previous studies have shown that the inhibitory effect of validamycin injection on *B. mori* and *T. castaneum*'s trehalase activities lasted for 21 and 7 d, respectively (Kono et al. 1993, Tatun et al. 2016). In addition, another competitive trehalase inhibitor, trehazolin, suppresses trehalase activity in the locust *Locusta migratoria* L. for approximately 30 d after the injection has been administered (Wegener et al. 2010). These examples are a testament to validamycin's long-lasting effects on tested insects (Jin and Zheng 2009). In addition, the results of the present study agree with the findings of other insect studies.

We found that the lethal effects of validamycin on adult *S. zeamais* were dependent on the concentration, with adult females being more susceptible than adult males. This finding aligns with that of a previous research report that showed that validamycin ingestion and injection both cause high mortality rates in *T. castaneum* during the larval and pupal stages, with pupae having a higher mortality rate than larvae (Tatun et al. 2016). In addition, in another study, validamycin induced high mortality rates in *Helicoverpa armigera* (Hübner), *Nilaparvata lugens* (Stål), and *Diaphorina citri* (Kuwayama) due to abnormalities that occurred during ecdysis (Tang et al. 2017, Adhav et al. 2018, Yu et al. 2021). Our study first demonstrated that validamycin decreases the reproduction success of adult *S. zeamais* based on the low number of F1 progeny in the validamycin-treated groups, which were concentration dependent. It is possible that the low mortality rate of *S. zeamais* after being treated with validamycin is due to the drug's interference with reproductive tissues such as the testes, ovary, and accessory glands; however, further research is necessary to confirm how validamycin affects the reproductive tissues at cytotoxicity and molecular levels. For example, the expression of genes involve in trehalose metabolism should be examined when the weevils are exposed to validamycin. Validamycin also appears to delay the developmental changes of

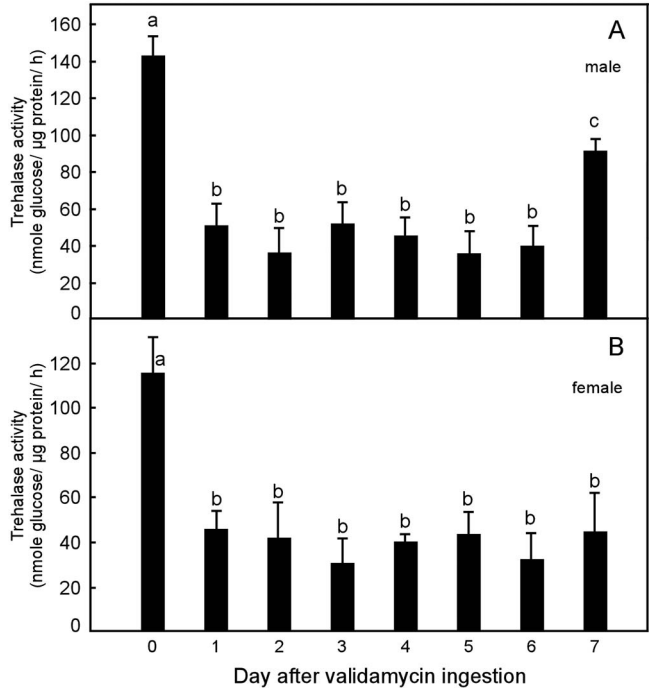

Fig. 7. Changes in *Sitophilus zeamais* trehalase activity after feeding 0.5 µg/ml validamycin during the adult stage. Trehalase activity was measured in male (A) and female (B) adults for 7 d. Trehalase activity is expressed in nanomoles of glucose per microgram of protein per hour. Error bars indicate SD, and the values labeled with different letters are significantly different ($P < 0.05$).

S. zeamais, which is similar to how it affects *Aedes aegypti* (L.) mosquitoes. In the mosquitoes, hatching, pupation, and eclosion were all significantly delayed in a concentration-dependent manner (Marten et al. 2020). Similarly, another study found that validamycin injection causes a longer developmental duration in *S. frugiperda* (Luo et al. 2022). Our study found that validamycin treatment reduced the size of *S. zeamais* larvae and pupae and also exhibited insecticidal properties in the adult and larval stages because we found dead larvae inside the artificial seeds treated with validamycin. The delay in the development of *S. zeamais* may be caused by a lack of glucose and carbon due to the inhibition of trehalase activity by validamycin. This inhibition negatively affected the insect's reproductive success, development, and survival rate; however, before using validamycin as an insect control agent, more research is needed to understand the impact of its inhibition of trehalase genes.

Acknowledgments

This work was supported by the revenue budget, School of Science, University of Phayao (PBTSC64011), awarded to O.W. The authors gratefully acknowledge the partial support of the Thailand

Science Research and Innovation Fund and the University of Phayao to N.T. (FF65-UoE010). This manuscript (no. 248733) has been edited by Proofed Inc (Dover, DE).

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