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Silica nanoparticle effect on population parameters and gene expression of an internal feeder, American serpentine leafminer

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Abstract

Insects quickly develop resistance to conventional chemical insecticides. The use of silica nanoparticles (SiO_2NPs) is a promising new approach for managing insect pests. The insects that have been studied are, in most cases, external feeders and internal leaf feeders have not been well studied. Here, we investigate the efficiency of SiO_2NPs in controlling the American serpentine leafminer *Liriomyza trifolii* (Diptera: Agromyzidae), a devastating insect pest of a wide range of crops. SiO_2NPs at concentrations of 50, 100, 200 and 400 mg/L compared to a control of distilled water were applied to intact *Phaseolus vulgaris* leaves by spraying to evaluate their effects, via the plant, on the number, survival, developmental/feeding speed, body mass/size and sex ratio of *L. trifolii* released after spraying. A qRT-PCR analysis was conducted to assess oxidative stress in *L. trifolii* based on the gene expression level of the two major antioxidant enzymes; catalase and superoxide dismutase. The number of mines, survival rate and puparia weight were lower than the control at the highest concentration of SiO_2NPs, while pupariation was delayed at most of the concentrations studied and puparium weight/size decreased at low concentrations. Sex ratio was not affected by SiO_2NPs. Gene expression levels of the antioxidant enzymes at the pupal stage were not significantly affected by SiO_2NPs at any concentration but some individuals showed up-regulation at low SiO_2NP concentrations, inferring development of resistance. This study demonstrated that *a priori* application of the highest concentration of SiO_2NPs to plants effectively reduces a leafminer population but that application of even the lowest concentration can decrease the life history trait values of surviving leafminers.

Keywords Silica nanoparticles • *Liriomyza trifolii* • *Phaseolus vulgaris* • qRT-PCR • Oxidative stress • Dose dependency

Introduction

To date, synthetic insecticides are the main strategy in the management of insect pests. Despite their efficacy, these chemical substances raise concerns of increasing insect resistance over successive generations. Moreover, the widespread and haphazard use of synthetic pesticides has negative effects on non-target organisms and poses a threat to human health. The use of nanoparticles (less than 100 nm in size in all three dimensions) in pest management can overcome the emerging problems of insecticide resistance by reducing the high-volume application of traditional chemical insecticides (Rani et al. 2014).

Silicon dioxide nanoparticles (SiO_2NPs) are the most popular among numerous types of nanomaterials (materials less than 100 nm in size at least in one dimension) and ensure definite interactions between molecules through a mesoporous structure carrying specific functional groups (Kaziem et al. 2017). SiO_2NPs are a stable and environmentally safe approach to pest management not only because of their versatility as an insecticide but also for improving the activity of other chemical insecticides. Neither the World Health Organization (WHO) nor the US Department of Agriculture have indicated any significant negative effects of SiO_2NPs on human health (El-Naggar et al. 2020). Depending on their application, size, and concentration, SiO_2NPs might be a viable alternative to conventional pesticides. SiO_2NPs are believed to interrupt insect physiological functions through respiratory blockage, cuticular damage, desiccation and injuries to the midgut

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epithelium (Caceres et al. 2019) in addition to altering enzymatic protection from oxidative stress. Studies that investigate the mechanisms by which SiO_2NPs affect the behavior, population parameters and gene expression of target organisms are critical in furthering our understanding of the entomotoxic effects of these nanoparticles and their applications in pest control.

The available literature on the entomotoxic effects of SiO_2NPs focuses on insect pests of stored grains, such as *Sitophilus oryzae* (Goswami et al. 2010; Debnath et al. 2011; El-Naggar et al. 2020), *Callosobruchus maculatus* (Rouhani et al. 2012; Arumugam et al. 2016), *Corcyra cephalonica* (Vani and Brindhaa 2013), *Rhizopertha dominica*, *Tribolium castaneum* and *Oryzaephilus surinamensis* (El-Naggar et al. 2020). More recently, entomotoxic effects have also been tested on insect pests in field settings, including the lepidopterans *Spodoptera littoralis* (El-Samahy et al. 2015; Ayoub et al. 2017), *Spodoptera litura*, *Achaea janata* (Rani et al. 2014), *Mythimna separata* (Mousa et al. 2014) and *Plutella xylostella* (Shoaib et al. 2018; Bilal et al. 2020), as well as the aphids *Lipaphis pseudobrassicae* (Debnath et al. 2010), *Aphis gossypii* (Pavitra et al. 2018), and the mealybug *Phenacoccus solenopsis* (Pavitra et al. 2018). These insects are all external feeders, whereas internal leaf feeders such as leafminers have not been well studied of SiO_2NP effects. It is reported that, as an intraspecific variation, internal feeding developmental stage (the larva) of seed feeders is less affected than external feeding stage (the adult) in a seed feeder (Rouhani et al. 2012). The American serpentine leafminer *Liriomyza trifolii* (Diptera: Agromyzidae) is a highly polyphagous species and one of the most damaging leafminers found in tropical and subtropical regions. *Liriomyza trifolii* larvae consume leaf mesophyll tissue, which interrupts the photosynthetic process and allows for the spread of diseases. This leads to a decrease in crop production, including production of the common bean *Phaseolus vulgaris* (Ibrahim 2008; Yıldırım et al. 2010).

To determine the usefulness of SiO_2NPs in controlling the leafminer *L. trifolii*, we investigated (1) the effect of SiO_2NPs on the number, survival, developmental/feeding velocity, body mass/size and sex ratio of developing *L. trifolii* with their larvae internally feeding on plant leaves treated with SiO_2NPs and (2) the expected genotoxicity induced by SiO_2NPs on the expression of antioxidant genes in *L. trifolii*. This study may be the first report on the entomotoxic effect of SiO_2NPs on internally feeding insects, dipteran leafminers.

Materials and methods

Silicon dioxide nanoparticles

A white powder of silicon dioxide (SiO_2) nanoparticles (NPs) [99.5% purity, 19.6 ± 5.8 nm (mean ± SD) in size and spherical in shape] were obtained from US Research Nanomaterials, Inc. (Houston, Texas, USA). A stock solution (1000 mg/L) of SiO_2NPs was prepared by dissolving the powder of SiO_2NPs in distilled water. This solution was sonicated for 30 min and centrifuged (2000 rpm, 25°C) for another 30 min to precipitate the non-dispersed agglomerated particles, which were filtered out of the solution using filter paper No. 2 (90 mm, Advantec, Japan). Then, four different concentrations of SiO_2NPs—50, 100, 200 and 400 mg/L—were prepared for investigating the toxic and genotoxic effects against the insect pest *L. trifolii*. The size and shape of SiO_2NPs were inspected using a high-resolution transmission electron microscope (TEM) (JEM-2100, JEOL Ltd.) at an accelerating voltage of 200 kV.

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Toxicity to the pest insect *L. trifolii*

Stock culture

A culture of *L. trifolii* was founded from a field-collected population in Fukuoka City, Japan and maintained under constant laboratory conditions (25 ± 2°C, 50 ± 10% RH and a 12L:12D photoperiod). Insects were reared inside transparent cuboid cages (70 × 50 × 50 cm) with a window (20 × 20 cm) on both sides and two windows (50 × 20 cm) on the back; all windows were covered with mesh to ensure sufficient ventilation. Two trays containing about 40 plants of the common bean *Phaseolus vulgaris* (a preferred host) [seeds obtained from Hokkaido, Japan by Nakahara Seed Co., Ltd. (Fukuoka, Japan)] were introduced daily. This allowed for continuous oviposition by *L. trifolii*, providing pupae in cocoons (puparia) to be used subsequently. *Phaseolus vulgaris* was planted weekly under long-day conditions (21–27°C and a 16L:8D photoperiod).

Population parameters

Two-week-old healthy *P. vulgaris* plants were sprayed with one of the prepared concentrations of SiO_2NPs (5 ml/plant) or with distilled water alone as a control treatment. Plants were sprayed using an ordinary drizzle (drop size of 1.05 ± 0.13 mm) at a distance of 15 cm to ensure a full coverage of both the upper and lower surfaces of the leaves (two leaves/plant). Plants were then left to dry at room temperature inside a transparent cylindrical cage (70 cm height × 25 cm diameter) covered with a fine mesh at the top in a randomized complete block design (RCBD) with six replications. After one hour, 48 h old male and female *L. trifolii* were released into the previously described cages (six for each treatment) to feed and lay eggs. After 24 h, the adults were removed and the eggs were allowed to complete their life cycle. To evaluate the toxic effect of SiO_2NPs, the following population parameters were recorded; the number of mines, larval survival rate, mine and pupariation time, larval feeding velocity, puparium weight/size, adult size and sex ratio.

Number of mines, larval survival rate and pupariation time

The total number of mines during the early larval stage on each day was counted for four days from the release of the adults. The number of puparia was counted every day for four days including the day the first puparium emerged. The larval survival rate was derived based on the number of puparia and the number of mines during the early larval stage.

Larval feeding velocity

One mine was randomly chosen for each replicate and digital pictures of the mine on the 2nd and 3rd days were captured. The length of the mines in both images were measured by dividing each mine (winding feeding tunnel, Ayabe et al. 2008) into short, straight lines (and by calibrating with the reference scale of each picture). Then, the larval feeding velocity was calculated as a measure of larval activity as

$$\text{Larval feeding velocity (LFV)} = \frac{L_2 - L_1}{\Delta T},$$

where L1 and L2 refer to the lengths of the mine on the 2nd and 3rd days, respectively, and ΔT is the time between the two images. One mine per replicate and six replicates per treatment were measured.

Puparium weight and size

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Five puparia (24 h old) per replicate were randomly selected and weighed together with a microbalance (AT-20, Mettler Toledo, Switzerland). The width of puparia was also measured for each of five puparia per replicate to the precision of 0.001 mm with a microscope (VH-5500, Keyence, Osaka, Japan). Afterwards, the puparia were stored in a freezer at −20 °C for future genetic analysis.

Adult size and sex ratio

Emerging adults were randomly collected from each replicate, frozen and sexed based on their genital morphology under a microscope. The numbers of females and males were counted and sex ratio (female ratio) was derived from them. The width of wings was also measured to the precision of 0.001 mm with a microscope, for each of five adults per replicate (sex unspecified).

Quantitative RT-PCR analysis of gene expression

The expression levels of two major antioxidant enzymes, catalase (*CAT*) and superoxide dismutase (*SOD*), in *L. trifolii* puparia (48 h old) as a response to the SiO_2NPs treatments were examined using qRT-PCR. Puparium was individually homogenized in the homogenizer (BHA-6, As One) with Isogen II (Nippon Gene) and zirconia beads (3,000 rpm, 1 min). Total RNA was extracted from the homogenate and used as a template for RT-PCR. The cDNAs were synthesized using SuperScript IV VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer’s instruction. The target genes were amplified using recombinant Taq DNA polymerase and the tracer EvaGreen (Biotium) with gene-specific primer sets obtained from FlyPrimerBank (Hu et al. 2013); catalase (CG6871): forward (F): 5´-GATGCGGCTTCCAATCAGTTG-3´ and reverse (R): 5´-GCAGCAGGATAGGTCCTCG-3´; superoxide dismutase 2 (CG8905): F: 5´-AAGTCGGGCAAACTGCAACT-3´ and R: 5´-GGACGCACGTTCTTGTACTG-3´. As a reference gene, β-actin was chosen and amplified with the following primer set: F: 5´- TTGTATTGGACTCTGGTGACGG-3´ and R: 5´- GATAGCGTGAGGCAAAGCATAA-3´ (Chang et al. 2017). Negative controls containing water instead of cDNA template were included for each primer set. The amplification plots were analyzed in the StepOnePlus real-time PCR system (Applied Biosystems) with the following cycling conditions: 94°C for 3 min followed by 40 cycles consisting of 94°C for 10 s, 60°C for 15 s, and 72°C for 15 s. Fluorescence readings were taken at the end of each cycle. The melting curve protocol contained 1 cycle at 95°C for 15 s and 60°C for 1 min. The temperature was increased from 60°C to 95°C at a rate of 0.3°C s^{-1}. The gene expression level was normalized by dividing by the mean gene expression level of the control. Three puparia were analyzed per treatment.

Statistical analysis

The following methods were applied to test the effect of different concentrations of SiO_2NPs on *L. trifolii*. The number of mines was analyzed using a generalized linear model with a Poisson distribution and a log link function and followed by a posthoc pairwise comparison with a Bonferroni correction. Larval feeding velocity was analyzed using a generalized linear model with a normal distribution and a log link function and followed by a posthoc pairwise comparison with a Bonferroni correction. Puparia weight and puparium width were analyzed using ANOVAs after confirming the normality of data distributions and followed by posthoc Dunnett tests for multiple comparisons with the control. Larval survival rate and sex ratio were analyzed using a logistic regression model with a logit link function and followed by a posthoc pairwise comparison

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with a Bonferroni correction. For oviposition and development time, dates of mine appearance and pupariation (sensored data) were analyzed using parametric survival analyses with the best-fit distributions (Weibull, log-normal, exponential, Frechet or log-logistic) and followed by a posthoc pairwise comparison with a Bonferroni correction. Kruskal-Wallis tests were used to compare gene expression levels. All statistical tests were performed using JMP 13.2.1.

Results

Toxicity to the insect pest *L. trifolii*

Number of mines

The number of mines representing the number of larvae was significantly different among SiO_2NP concentrations (likelihood ratio $\chi^2_4 = 58.29$, $P < 0.0001$). Smaller numbers of mines were observed than the control at a concentration of 400 mg/L (Fig. 1).

Larval survival rate

The larval survival rate to adult stage was different among SiO_2NP concentrations (likelihood ratio $\chi^2_4 = 19.97$, $P = 0.0005$) and was significantly lower than the control at a concentration of 400 mg/L (Fig. 1).

Mine appearance time and pupariation time

Time taken for mine appearance was significantly different among SiO_2NP concentrations (Weibull distribution, likelihood ratio $\chi^2_4 = 31.95$, $P < 0.0001$) and longer than the control at concentrations of 100 and 200 mg/L (Fig. 2). The date of pupariation was significantly different among SiO_2NP concentrations (log-normal distribution, likelihood ratio $\chi^2_4 = 19.64$, $P = 0.0006$) and longer than the control at concentrations of 50, 100 and 400 mg/L (Fig. 2).

Larval feeding velocity (LFV)

The larval feeding velocity was significantly different among SiO_2NP concentrations ($\chi^2_4 = 16.46$, $P = 0.003$). Feeding velocity was significantly lower than the control at 50 mg/L.

Puparium weight and size

The puparia weight (as a mean of five late-stage puparia) showed significant differences among SiO_2NPs concentrations ($F_{4,25} = 8.38$, $P = 0.0002$) and was lower than the control at concentrations of 50, 100 and 400 mg/L (Fig. 3). The puparium width was also different among SiO_2NPs concentrations ($F_{4,145} = 14.07$, $P < 0.0001$) and was shorter than the control at 50 mg/L (Fig. 3).

Adult size and sex ratio

The adult wing width was different among SiO_2NPs concentrations ($F_{4,139} = 4.08$, $P = 0.004$) but a posthoc test did not detect any difference from the control (Fig. 3). Sex ratio (female ratio) (0.636 ± 0.0680, mean ± SE, $n = 19$) was not significantly different among SiO_2NP concentrations (likelihood ratio $\chi^2_4 = 5.06$, $P = 0.282$).

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Gene expression

No concentration-dependent impacts of SiO_2NPs were observed on the expression levels of catalase or superoxide dismutase genes (catalase, $H_{3,3,3,3,3} = 3.33$, $P > 0.1$; superoxide dismutase, $H_{3,3,3,3,3} = 4.77$, $P > 0.1$). This is due to the large variation in gene expression levels among individuals (surviving pupae) in each treatment (Fig. 4). At concentrations of 50 and 100 mg/L, some pupae showed up-regulated gene expression for catalase compared to the control, whereas the gene expression levels of superoxide dismutase were relatively constant across the different SiO_2NP concentrations.

Discussion

The number of mines decreased at high concentrations of SiO_2NP. It approximates the number of young larvae feeding in leaves. The decrease in the number of mines may result from (1) decreased female oviposition preference for, (2) higher mortality of adults on, and/or (3) higher mortality of eggs in the SiO_2NP-treated plant. The larval survival rate and puparia weight were also lower than the control at the highest concentration of SiO_2NPs, while pupariation was delayed at most of the concentrations studied and feeding velocity and puparium weight/size decreased at low concentrations. Offspring sex ratio was not affected by SiO_2NPs, indicating no biased mortality between females and males. This study demonstrated that the application of the highest concentration (400 mg/L) of SiO_2NPs to soybean prior to infestation effectively reduces a leafminer population but that even the lowest concentration (50 mg/L) can decrease the life history traits of surviving leafminers, especially during the larval stage (Fig. 3).

Expression levels of antioxidant genes were not significantly different among SiO_2NP concentrations but some individuals showed up-regulation compared to the control. The amount of time since the initial exposure to SiO_2NPs may have allowed for the development of resistance in surviving immature *L. trifolii*. These individuals might have had a genetic variant or sex-specific difference (Muller 2018) that allowed for higher resistance against the negative effects of feeding on SiO_2NP-treated plant tissues as larvae, which needs future investigation.

When applied externally, SiO_2NPs kill insects by dehydration (Ayoub et al. 2017; Shoaib et al. 2018). Other possible mechanisms of SiO_2NP-induced insect mortality include the blockage of spiracles and tracheae (part of the respiratory system) or damage to the surface wax on the outer cuticle of the insect by sorption (physisorption) and abrasion (Rastogi et al. 2019). In this study, however, SiO_2NPs did not contact the outer cuticle of the insect *L. trifolii* since the nanoparticle solution was applied to the plant prior to infestation by *L. trifolii*. Silicon absorbed and deposited in plant epidermal tissues (e.g. cell walls, lumen, intracellular space and trichomes, Cooke and Leishman 2011) can increase the rigidity of the tissue, thereby increasing its mechanical resistance to herbivory and causing abnormal level of wear on mandibles of the insects that feed on it (Painter 1951; Sasamoto 1955; Takahashi 1995; Keeping and Meyer 2002; Reynolds et al. 2009; Massey and Hartley 2009). Silica may also cause impairment of the digestive tract in insect herbivores (Smith 1969). Additionally, the application of silicon can enhance natural defense system of the host plant in response to herbivory (Coscun et al. 2019; Hall et al. 2019; Leroy et al. 2019) by producing increased quantities of flavonoids and phenolic acids (Fawe et al. 1998) and by promoting jasmonate-mediated defenses (Ye et al.

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2013). Similar effects but with possibly faster absorption and consequent swift biological response are expected for nano-size silica.

Based on our results, applying a low concentration (50 mg/L) of SiO_2NPs prior to infestation to plants can decrease the life history traits of the leafminer *L. trifolii* population while avoiding negative impacts on the plant *P. vulgaris* (promoted seedling growth at 400 mg/L, the lowest concentration studied, but negative effects on growth at 2000 and 4000 mg/L, Sharifi-Rad et al. 2016). However, SiO_2NPs even at this level of concentration could be accumulated and toxic to human cells (Al-Rawi et al. 2011; Ariano et al. 2011; but see Kettiger et al. 2015). To effectively reduce the population size of the leafminer, a higher concentration (400 mg/L) need to be applied *a priori* to plants. For a more efficient and safer use of SiO_2NPs at lower concentrations on *L. trifolii*, future studies should investigate the effects of directly applying SiO_2NPs to its externally feeding stage, adults.

Author Contribution Statement

Conceptualization, A.F.T., O.A.G. and M.T.; investigation, A.F.T., R.F. and M.H.; statistical analysis, M.T.; writing—original draft preparation, A.F.T., M.T. and R.F.; writing—review and editing, M.T. and O.A.G.; visualization, A.F.T., M.T. and R.F.; supervision, M.T., O.A.G. and M.F.M.E. All authors have read and agreed to the published version of the manuscript.

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Figure captions

Fig. 1 The number of mines (mean ± SE) and larval survival rate (mean) of the leafminer *Liriomyza trifolii* at different concentrations of SiO_2NPs. An asterisk indicates a significant difference from the control

Fig. 2 Mine appearance time and pupariation time (mean ± SE) of the leafminer *Liriomyza trifolii* at different concentrations of SiO_2NPs. An asterisk indicates a significant difference from the control

Fig. 3 Weight of five puparia, puparium width and adult wing width (mean ± SE) of the leafminer *Liriomyza trifolii* at different concentrations of SiO_2NPs. An asterisk indicates a significant difference from the control

Fig. 4 Gene expression levels of antioxidant enzymes for the leafminer *Liriomyza trifolii* at different concentrations of SiO_2NPs. Means are presented by horizontal bars

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Fig. 1

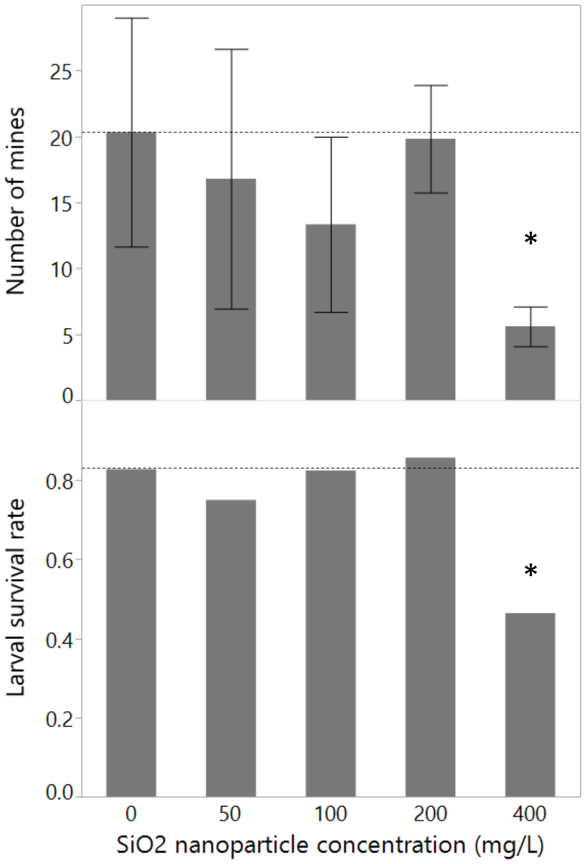

Fig. 2

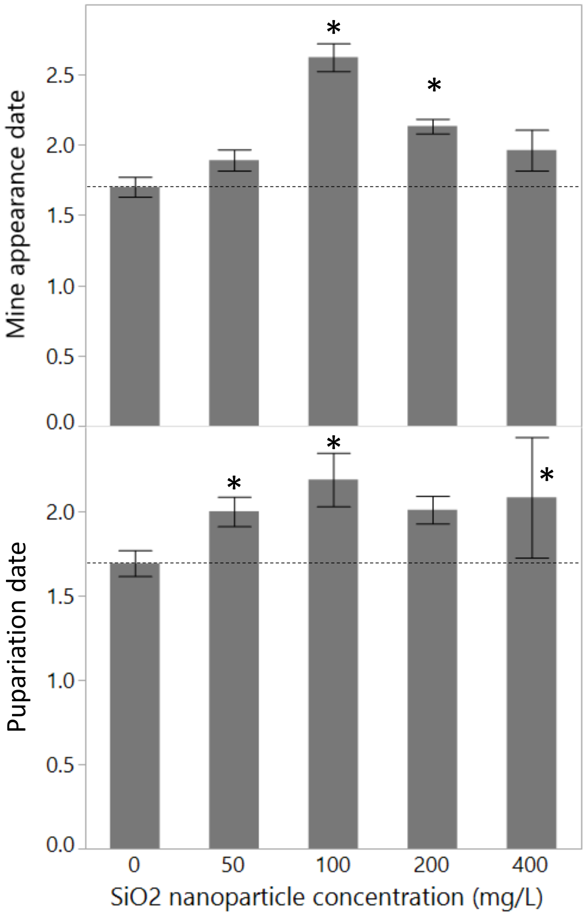
Mine appearance date
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pupariation date
2.0
1.5
1.0
0.5
0.0
*
*
*
0
50
100
200
400
SiO2 nanoparticle concentration (mg/L)

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Fig. 3

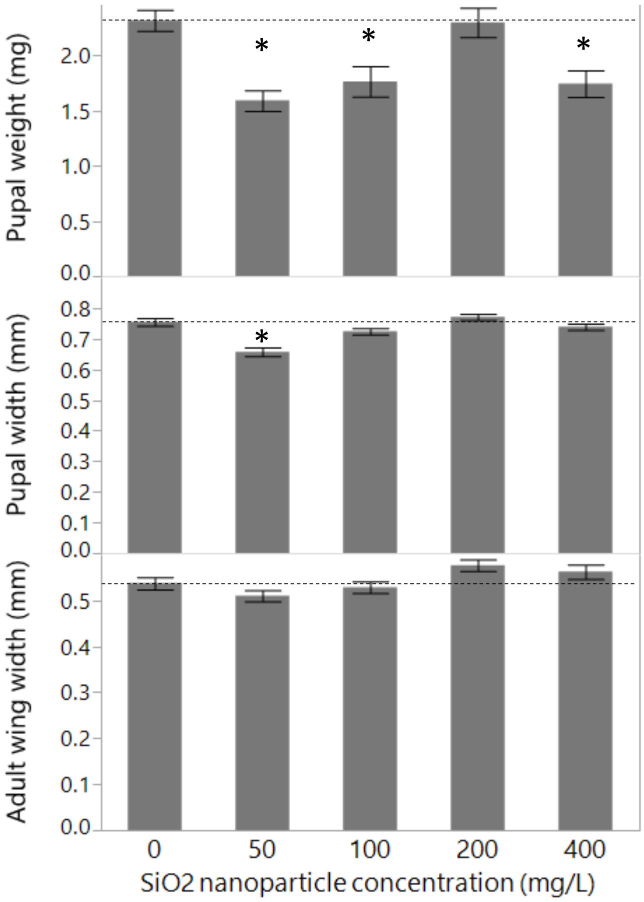

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Fig. 4