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The hatching-stimulation activity of solanoeclepin A toward the eggs of *Globodera* (Tylenchida: Heteroderidae) species

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Abstract Plant-parasitic nematodes often interact with their host plants via chemical compounds for successful invasion. In cyst nematodes, hatching requires chemicals known as hatching factors that are released from host roots. Previous studies succeeded in identifying and synthesizing solanoeclepin A (SEA) as a host-derived hatching factor for *Globodera rostochiensis* (Wollenweber) Behrens and *Globodera pallida* (Stone) Behrens (Tylenchida: Heteroderidae). However, no published data were available about the hatching response of *Globodera* species, other than *G. rostochiensis*, toward SEA. In this study, we tested the hatching responses of *G. rostochiensis*, *G. pallida*, *Globodera ellingtonae* Handoo, Carta, Skantar, and Chitwood, *Globodera tabacum* (Lownsbery and Lownsbery) Behrens, and *Globodera artemisiae* (Eroshenko and Kasachenko) Behrens (Tylenchida: Heteroderidae) toward SEA by exposing eggs of each species to 0.001, 0.01, 0.1, 1, 10, and 100 ppb of SEA and observing their hatching response. SEA stimulated the hatching of Solanaceae-parasitic *Globodera* species (*G. rostochiensis*, *G. pallida*, *G. ellingtonae*, and *G. tabacum*). The optimal concentration of SEA for hatching was 0.01–10 ppb. However, the hatching activity of *G. pallida* and *G. ellingtonae* was lower than that of *G. rostochiensis* and *G. tabacum*. SEA showed no effects on *G. artemisiae* which is parasitic to *Artemisiae* plants.

Keywords *Globodera tabacum* · hatching factor · potato cyst nematodes · Solanaceae

Introduction

Globodera rostochiensis (Wollenweber) Behrens and *Globodera pallida* (Stone) Behrens (Tylenchida: Heteroderidae) are significant pests of potato. These species cause poor plant growth, and reduced tuber number and size (Trudgill et al. 1975a, b). Although loss of potato yields due to these species are influenced by multiple factor (soil type, cultivar, weather, site etc.), more than 50 % loss in total potato yield have been reported (Trudgill 1986; Trudgill et al. 2014). They can also attack other Solanaceae crops and have been problematic for tomato production in Europe (Ellis 1968; Ellis and Smith 1971; Graham 1966; Hesling and Ellis 1972; Trifonova et al. 1995). Recently, another *Globodera* species parasitic to potato, *Globodera ellingtonae* Handoo, Carta, Skantar, and Chitwood (Tylenchida: Heteroderidae), was discovered in potato production fields in USA (Handoo et al. 2012; Skantar et al. 2011) and is also parasitic to tomato (Peetz et al. 2019). These three *Globodera* species, *G. rostochiensis*, *G. pallida*, and *G. ellingtonae*, are collectively known as potato cyst nematodes.

Another Solanaceae-parasitic *Globodera* species, the tobacco cyst nematode, *Globodera tabacum* (Lownsbery and Lownsbery) Behrens (Tylenchida: Heteroderidae), is problematic for worldwide tobacco production. *G. tabacum* causes stunting and reduced leaf mass in tobacco plants (LaMondia 1988; Parkunan et al. 2009) and reduces tobacco yield by up to 45 % (LaMondia 1995a). The host range of *G. tabacum* also includes tomato and eggplant (Harrison and Miller 1969; Lownsbery 1953).

Control of these *Globodera* species is essential for production of Solanaceae crops. However, currently used nematicides such as fumigants are going to be

withdrawn in some countries because of their burden on the environment (Chitwood 2002, 2003). Therefore, environmentally friendly techniques to control *Globodera* pests are required.

The life cycle of *Globodera* species is synchronized with their hosts for successful invasion (Masler and Perry 2018; Perry 1989). Eggs of *Globodera* species can survive in soil for long periods contained within the cyst, a protective shell (Moens et al. 2018). However, in the presence of host plants, large numbers of eggs hatch in response to hatching factors (HFs) secreted by host roots (Devine et al. 1996; LaMondia 1995b). Then, hatched juveniles invade host plant roots and deprive the plant of nutrients. Host-derived HFs could be used to induce hatching in the absence of host plants, resulting in the death of cyst nematodes without further environmental damage (Chitwood 2002, 2003). Therefore, many studies have focused on the ecology of *Globodera* hatching and the identification of HFs from host root diffusates (reviewed in Masler and Perry 2018; Perry 1998, 2002).

A previous study isolated Solanoeclepin A (SEA), a powerful host-derived HF for potato cyst nematodes, and identified its structure (Mulder et al. 1996). Moreover, a recent study succeeded in synthesizing SEA and reported its remarkable hatching-stimulation activity toward *G. rostochiensis* (Tanino et al. 2011). However, no published data are available about the hatching responses of *G. pallida* and *G. ellingtonae* toward SEA. We hypothesize that *G. tabacum* might also respond to SEA because this species is closely related to potato cyst nematodes and is Solanaceae-parasitic. Clarifying the response of *G. pallida*, *G. ellingtonae*, and *G. tabacum* toward SEA could improve understanding of their hatching ecology and this

may facilitate the development of control techniques for these species. In addition, it is unknown whether other *Globodera* species, which are not parasitic to Solanaceae plants, respond to SEA.

The aim of this study was to clarify whether potato cyst nematodes (i.e., *G. rostochiensis*, *G. pallida*, and *G. ellingtonae*), *G. tabacum*, and other *Globodera* species parasitic to non-Solanaceae plants (we used *Globodera artemisiae* (Eroshenko and Kasachenko) Behrens (Tylenchida: Heteroderidae), parasitic to *Artemisiae* spp.) hatch in response to SEA.

Materials and Methods

Nematodes

Each *Globodera* species was reared on its host plants. The origin and the rearing conditions of each species are described in Table 1. Once the plants had died, the soil containing the cysts was dried and stored at 4 °C for at least 9 months.

Preparation of chemicals

To ensure the viability of nematodes used in this study, we used tomato root diffusate (TRD) for the hatching test of *G. rostochiensis*, *G. pallida*, *G. ellingtonae*, and *G. tabacum* and mugwort root diffusate (MRD) for that of *G. artemisiae*. To obtain TRD, seeds of tomato cultivar Doctor-K were planted into polyethylene pots (15 cm diam.) containing culture soil (0.374 g N, 1.485 g P_2O_5, 0.242 g K_2O, 0.165 g MgO per kg; Hokusan Co., Ltd., Hokkaido, Japan). The plants were grown in a greenhouse for 5

weeks at a mean temperature of 25 °C. After that, the roots of each plant were gently washed, immersed in 300 mL of distilled water, and kept at room temperature (approximately 20 °C) for 24 h with aeration. The water was filtered using no. 3 filter paper (Advantec®, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and a 0.45 μm syringe filter (Sartorius AG, Göttingen, Germany) to remove debris and microorganisms. The resulting TRD was held at −25 °C until required. To obtain MRD, young plants of Japanese mugwort (*Artemisia indica* var. *maximowiczii*) grown from cuttings were planted into 15 cm diam. polyethylene pots containing culture soil and grown in a greenhouse for 4 weeks. Subsequently, MRD was prepared and stored in the same way as for TRD. Just prior to use, the TRD and MRD were diluted in distilled water to 40 % and 80 %, respectively.

Standard SEA, synthesized according to the method of Tanino et al. (2011) and dissolved in ethanol at a concentration of 10^{-4} g mL^{-1}, was diluted to 0.002, 0.02, 0.2, 2, 20, and 200 ppb in distilled water. SEA solutions were stored in a refrigerator until immediately before use in assays.

Hatching test

The cysts of each species were isolated from the soil according to the method described by Shepherd (1986) with some modifications. Dried soil (50–100 g) containing cysts was put into a 3 L plastic beaker. Approximately 3 L of water discharged from a shower nozzle was poured into the beaker while stirring. After a few minutes, the floating debris and cysts were carefully decanted into a pair of sieves (850 μm over 212 μm aperture). Water pouring and decanting were repeated several times. The contents of the

upper (850 μm) sieve were washed so that all the cysts passed into the lower (212 μm) sieve. After that, cysts on the lower sieve were collected to one edge of the sieve by using water discharged from a shower nozzle and transferred to a no. 3 filter paper on a glass funnel (90 mm diam.) by washing. The cysts were picked out from the isolate under a binocular and incubated in distilled water for 2–3 weeks at 20 °C. However, because *G. pallida* is well adapted to lower temperatures (Robinson et al. 1987), the cyst incubation and hatching test (described later) for *G. pallida* were performed at 16 °C. Subsequently, an egg suspension was prepared by crushing the cysts and removing dead eggs and hatched juveniles as completely as possible. Tween 20 (final concentration 0.01 %) was added to the resulting egg suspension to prevent nematode eggs from adhering to the inside wall of the plastic pipettes.

The egg suspension (100 μL, containing approximately 100 eggs) was added to each well of 96-well Nunclon™ Delta plates (Thermo Fisher Scientific, Waltham, MA, USA). One hundred microliters of distilled water, SEA solution (0.002–200 ppb), or root diffusate (TRD or MRD) were added to the wells containing eggs. Thus, nematodes were exposed to final concentrations of SEA of 0.001, 0.01, 0.1, 1, 10, and 100 ppb. Each plate was incubated at 16 °C (*G. pallida*) or 20 °C (all other species). We counted the hatched juveniles and unhatched eggs in each well at 14 days after treatment using a stereomicroscope. Each treatment was replicated 4 times.

As the synthetic standard of SEA was dissolved in ethanol, the effects of ethanol on hatching were also tested. To confirm that ethanol does not induce hatching, we treated eggs of each *Globodera* species with distilled water or ethanol solutions (0.01, 0.1, 1, 10, 100, and 1000 ppm, corresponding to the 0.001, 0.01, 0.1, 1, 10, and

100 ppb SEA solutions, respectively). In addition, to test whether ethanol inhibited the hatching-stimulation activity of SEA, we treated eggs of each *Globodera* species with mixture solutions of SEA (1 ppb) and ethanol (10, 100, or 1000 ppm), or water. At 14 days after treatment, the hatched juveniles and unhatched eggs were counted as described above. Each treatment was replicated 3–4 times.

Statistical analysis

The assay data were analyzed using a generalized linear mixed model (GLMM) with a binomial error distribution and logit link function. A model was constructed using hatching probability (i.e., number of hatched juveniles versus that of unhatched eggs) as a response variable, treatment as a fixed effect, and egg group (i.e., replicate) as a random effect. The effect of each treatment was tested using the likelihood-ratio chi-square test. We also conducted Tukey's *post hoc* test for pairwise comparisons among treatments. The program packages lme4 v. 1.1.21 and multcomp v. 1.4.12 in R software v.3.4.2 (R Development Core Team 2017) were used for statistical analyses.

Results

G. rostochiensis

The treatments (water, SEA, or TRD) significantly affected the hatching ratio (Fig. 1a; $\chi^2 = 809.55$, df = 7, $P < 0.001$). The hatching ratios of TRD-treated eggs and SEA-treated eggs (at all SEA concentrations tested) were significantly higher than that of water-treated eggs. The optimum SEA concentration range for hatching was 0.1–10

ppb, and the hatching-stimulation activity of these SEA concentrations was equivalent to or significantly higher than that of TRD.

G. pallida

The treatments significantly affected the hatching ratio (Fig. 1b; $\chi^2 = 783.07$, df = 7, $P < 0.001$). The hatching ratio of TRD-treated eggs was significantly higher than that of water-treated eggs. The hatching ratios of the eggs treated with 0.1–100 ppb of SEA were also significantly higher than that of water-treated eggs. There were no significant differences between the hatching ratio of the eggs treated with 0.001 and 0.01 ppb of SEA and that of water-treated eggs. Although the optimum SEA concentration range for hatching was 1–10 ppb, the hatching-stimulation activity of these SEA concentrations was significantly lower than that of TRD.

G. ellingtonae

The treatments significantly affected the hatching ratio (Fig. 1c; $\chi^2 = 227.23$, df = 7, $P < 0.001$). The hatching ratio of TRD-treated eggs was significantly higher than that of water-treated eggs. The hatching ratios of the eggs treated with 0.01–100 ppb of SEA were also significantly higher than that of water-treated eggs. There was no significant difference between the hatching ratio of the eggs treated with 0.001 ppb of SEA and that of water-treated eggs. Although the optimum SEA concentration range for hatching was 0.1–100 ppb, the hatching-stimulation activity of these SEA concentrations was lower than that of TRD.

G. tabacum

The treatments significantly affected the hatching ratio (Fig. 1d; $\chi^2 = 802.47$, df = 7, $P < 0.001$). The hatching ratios of TRD-treated eggs and SEA-treated eggs (at all SEA concentrations tested) were significantly higher than that of water-treated eggs. The optimum SEA concentration range for hatching was 0.01–10 ppb, and the hatching-stimulation activity of these SEA concentrations was equivalent to that of TRD.

G. artemisiae

The treatments (water, SEA, or MRD) significantly affected the hatching ratio (Fig. 1e; $\chi^2 = 653.48$, df = 7, $P < 0.001$). The hatching ratio of MRD-treated eggs was significantly higher than that of water-treated eggs. However, there were no significant differences between the hatching ratios of SEA-treated eggs and that of water-treated eggs.

Effect of ethanol on hatching

For each species, no significant differences were observed between the hatching ratio of water-treated eggs and that of eggs treated with ethanol solution at any concentrations tested (data not shown). Although, in any species tested, the hatching ratios of eggs treated with mixture solutions of SEA and ethanol were significantly higher than that of water-treated eggs, the concentration of ethanol in solutions containing SEA did not significantly affect the hatching ratio (data not shown). *G. artemisiae* was not used for these tests because its hatching ratio in SEA solution did not significantly differ from

225 that in water.

226

227 **Discussion**

228

229 Tanino et al. (2011) succeeded in synthesizing SEA and described its remarkable
230 hatching-stimulation activity toward *G. rostochiensis*. The present study showed that
231 SEA stimulates hatching not only of *G. rostochiensis* but also of *G. pallida*, *G.*
232 *ellingtonae*, and *G. tabacum*. However, hatching of *G. artemisiae*, which is parasitic on
233 Asteraceae plants, was not stimulated by SEA. These results suggest that SEA acts as a
234 HF for *Globodera* species parasitic on Solanaceae plants. Solanaceae-parasitic
235 *Globodera* species are monophyletic and thought to be native to the Andes region
236 (Subbotin et al. 2020). Presumably, their common ancestor might have become
237 responsive to SEA, or its analogs, released specifically from Solanaceae roots grown in
238 the Andes. Furthermore, the hatching-stimulation activity of HFs is thought to involve
239 receptor-ligand interactions (Devine et al. 1996). Solanaceae-parasitic *Globodera*
240 species might possess similar HF-receptors.

241 The hatching ratio of *G. rostochiensis* in 0.1–10 ppb SEA was equal to or
242 greater than that in TRD. *G. tabacum* also responded similarly to 0.01–10 ppb SEA and
243 TRD. These results suggest that SEA acts as the main HF for *G. rostochiensis* and *G.*
244 *tabacum*. Presumably, the HF-receptor of these two species might have a high affinity
245 for SEA. In contrast, the hatching ratio of *G. pallida* and *G. ellingtonae* in SEA, at all
246 the concentrations tested, was lower than that in TRD for each species. This
247 demonstrated that not all potentially hatchable eggs of these species did actually hatch

248 in SEA. These results suggest that TRD contains other compounds that induce a high
249 hatching response in *G. pallida* and *G. ellingtonae*. Previous research has also suggested
250 that *G. rostochiensis*-preferred HFs were different from *G. pallida*-preferred HFs
251 (Devine and Jones 2003), and this is consistent with our results. The structure of the
252 HF-receptors of *G. pallida* and *G. ellingtonae* might differ slightly from those of *G.*
253 *rostochiensis* and *G. tabacum*. Several studies reported that solanaceae-parasitic
254 *Globodera* species form two clades: *G. tabacum*, *G. rostochiensis*, and *G. ellingtonae*;
255 *G. pallida* and *G. mexicana* (not used in this study), and the former clade contains two
256 subclades: *G. tabacum* and *G. rostochiensis*; *G. ellingtonae* (Lax et al., 2014; Subbotin
257 et al. 2011; Zasada et al., 2015). Therefore, their hatching responses toward SEA might
258 be relevant to their phylogenetic distances.

259 The optimal concentration of SEA for hatching was substantially 0.01–10 ppb
260 for all species in our study except for *G. artemisiae*. Glycinoeclepin A (GEA) is well
261 known as a HF for soybean cyst nematode, *Heterodera glycines* Ichinohe (Tylenchida:
262 Heteroderidae) (Masamune et al. 1982) and induce high hatching activity of *H. glycines*
263 at the concentrations of 0.1–1 nM (approximately 0.05–0.5 ppb) (Nonaka et al. 2016).
264 Therefore, the optimal concentrations of SEA for hatching correspond to those of GEA.

265 One hundred ppb SEA showed relatively low hatching-stimulation activity. We
266 recognized the possibility that the solvent of SEA (i.e., ethanol) might inhibit hatching
267 at higher concentrations. However, our results showed that increasing ethanol
268 concentration in SEA solutions did not affect the hatching ratios of *Globodera* eggs. In
269 insulin and insulin-receptor interaction, an elevated level of insulin causes a decrease of
270 the receptor, thereby reducing insulin sensitivity (Gavin et al. 1974; Krupp and Lane

271 1981). An analogous phenomenon might occur between SEA and HF-receptors. To test
272 this hypothesis, clarification of the HF-receptor and its response toward SEA may be
273 needed.

274 The utilization of HFs was previously proposed as a technique for control of
275 cyst nematodes (Perry 1998, 2002). A previous study showed that application of SEA
276 (100 μg m^{-2}) to a field reduced *G. rostochiensis* density by 85 % (unpublished data),
277 demonstrating that SEA could be used for effective chemical control of *G. rostochiensis*.
278 In this study, not only *G. rostochiensis* but also *G. tabacum* hatched well in SEA,
279 suggesting that SEA could also be an effective agent for control of *G. tabacum*.
280 However, SEA may not be efficient enough to control *G. pallida* and *G. ellingtonae*
281 because their hatching activity in SEA was relatively low. Further research is required to
282 elucidate the main HFs for *G. pallida* and *G. ellingtonae*.

283

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293

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FIGURE CAPTION

Fig. 1 Hatching ratios (number of hatched juveniles per total number of eggs) of (a) *Globodera rostochiensis*, (b) *G. pallida*, (c) *G. ellingtonae*, (d) *G. tabacum*, and (e) *G. artemisiae* eggs, treated with water, SEA (0.001–100 ppb), or root diffusates (mean ± SE, $N = 4$ each). The values with same letters are not significantly different (GLMM, likelihood ratio chi-square test followed by Tukey's test, $P > 0.05$)

403 **Table 1** The origin and rearing conditions of *Globodera* species used in this study

Species	Pathotype	Origin	Rearing condition
G. rostochiensis	Ro 1	Hokkaido, Japan	Reared on the potato cultivar Irish Cobbler[a] for three months in a greenhouse at a mean temperature of 20 °C
G. pallida	Pa 3	Hokkaido, Japan	Reared on the potato cultivar Pearl Starch[b] for three months in a greenhouse at a mean temperature of 18 °C
G. ellingtonae	-	USA	Reared on the potato cultivar Irish Cobbler[a] for two months in a phytotron chamber (LH-305S, Nihon Ika Co., Ltd., Osaka) at 18 °C
G. tabacum	-	Kochi, Japan	Reared on *Solanum integrifolium* 'Akanasu (Hiranasu)' for three months in a greenhouse at a mean temperature of 25 °C
G. artemisiae	-	Nagasaki, Japan	Reared on *Artemisia indica* var. *maximowiczii* for six months in a greenhouse at a mean temperature of 25 °C

404 [a]Potato cultivar Irish Cobbler has no potato cyst nematodes resistant gene

405 [b]Potato cultivar Pearl Starch has a resistant gene *H1*

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407 **Fig. 1**

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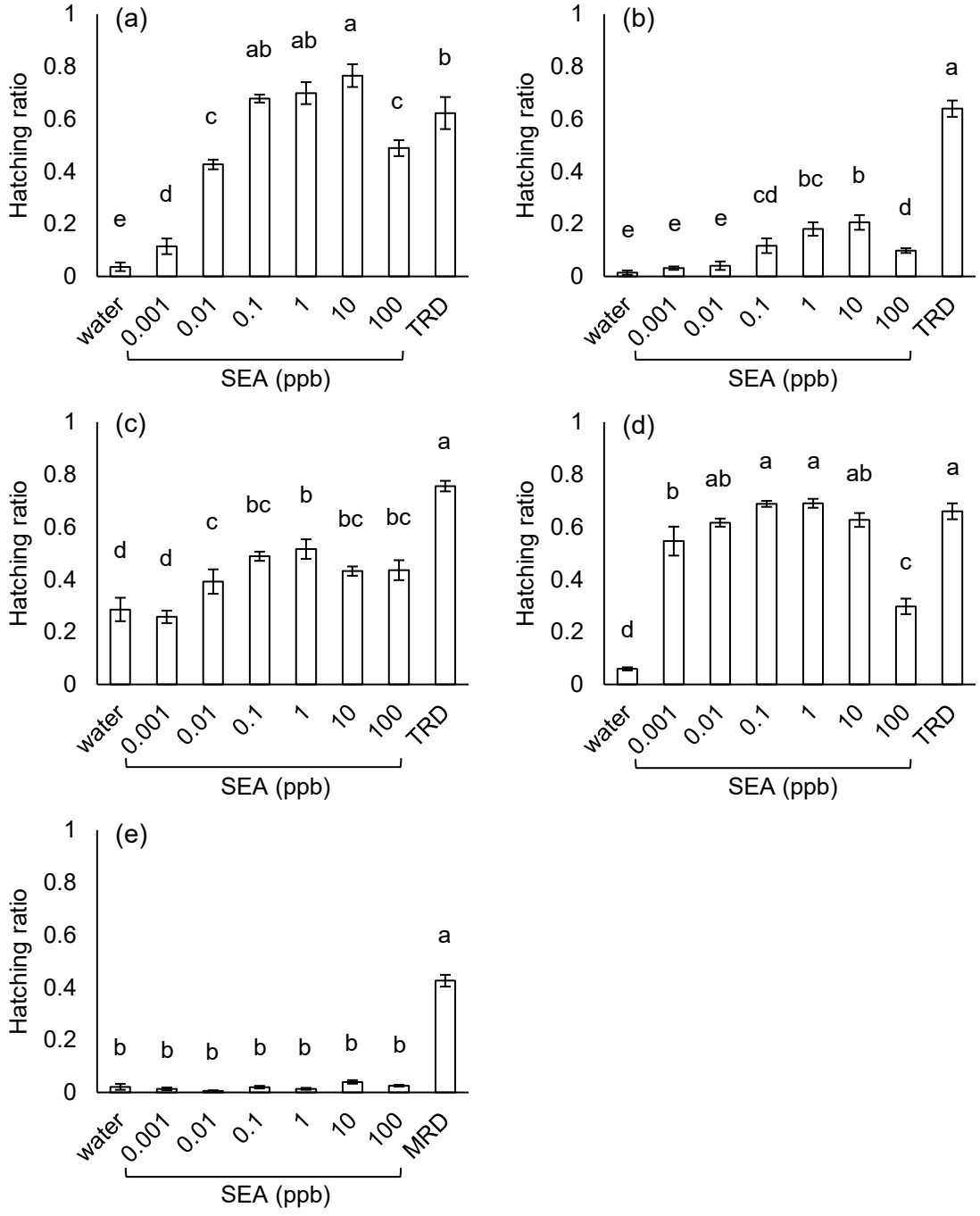

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