

1 **Species-specific elicitors induce tea leaf to arrest the endoparasitoid *Ascogaster***
2 ***reticulata* (Hymenoptera: Braconidae)**

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18

19 **Abstract**

20 *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae) is an egg-larval endoparasitoid

21 of the smaller tea tortrix, *Adoxophyes honmai* Yasuda. Recent studies have examined

22 tritrophic interactions among *Camellia sinensis*, *A. honmai*, and *A. reticulata*, but the effect of

23 non-host insects on the induction of tea plant that may affect foraging behaviour of *A.*

24 *reticulata* remains unclear. In this study, we selected two non-host insects, *Homona*

25 *magnanima* Diakonoff and *Ostrinia furnacalis* (Guenée), as representative species in our

26 bioassays. Tea leaves were treated with homogenized female reproductive tissues of a non-
27 host insect for comparison with untreated leaves in a choice test. Residence times of
28 parasitoids on both leaves were recorded. The parasitoids seemed to prefer walking on leaves
29 treated with homogenates of *H. magnanima* over untreated leaves, but the difference in
30 residence times was not significant. In contrast, its residence time on leaves treated with
31 homogenates of *O. furnacalis* was significantly shorter than that on untreated leaves. Thus,
32 the induction of tea leaf surface chemicals may differ among moth species, which may
33 produce different types of elicitors. This difference may, in turn, affect the host-searching
34 behaviour of *A. reticulata*.

35

36 **Keywords:** *Adoxophyes honmai*, *Homona magnanima*, *Ostrinia furnacalis*, tea leaf
37 induction, host-searching behaviour

38

39 **Introduction**

40 The smaller tea tortrix, *Adoxophyes honmai* Yasuda (Lepidoptera: Tortricidae), is a common
41 pest of tea plants (*Camellia sinensis* L.) in Japan. Piyasaengthong, Sato, Kinoshita, and
42 Kainoh (2016a) reported that female moths prefer to oviposit on the abaxial side of tea
43 leaves, and the young larvae prefer to feed on young shoots and leaves. These preferences
44 explain why damage caused by this species can be found on many parts of tea plants,
45 especially on their leaves, affecting the productivity, quantity, and quality of tea. To prevent
46 such damage, farmers need to spray insecticides in tea fields several times a year. The
47 impacts of decades of insecticide use on human health and the environment are a concern.
48 Therefore, to reduce the use of insecticides, other methods have been introduced for
49 controlling *A. honmai* populations, such as the application of mating disruptors in Shizuoka
50 Prefecture since 1983 (Tamaki, Noguchi, and Sugie, 1983). Moreover, natural enemies play
51 an important role as biological control agents (Hazarika, Bhuyan, and Hazarika, 2009).

52 *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid
53 found in Japanese tea fields, can control *A. honmai* populations by parasitizing the host and
54 finally killing it in the fourth instar (Watanabe, 1967; Kawakami and Kainoh, 1986). Larval
55 mortality caused by *A. reticulata* parasitism was 38% (Nakai, Takeda, and Kunimi, 1997).
56 Kainoh, Hiyori, and Tamaki (1982) reported that host-searching behaviour of *A. reticulata*
57 was normally stimulated by host (*A. honmai*) kairomones, such as those from moth wing
58 scales or excrement. In addition to using host kairomones, *A. reticulata* females can learn and
59 discriminate chemicals from various plant species as cues to locate their hosts (Kainoh, 1997;
60 Seino and Kainoh, 2008; Seino, Shoji, and Kainoh, 2010). Moreover, *A. reticulata* can detect
61 changes in surface chemical(s) on the adaxial side of tea leaves to locate *A. honmai* egg
62 masses deposited on the abaxial side (Deshpande and Kainoh, 2012; Piyasaengthong,
63 Kinoshita, Sato, and Kainoh, 2016b). Plant volatiles that are induced specifically by insect

64 herbivore oviposition are called Oviposition-Induced Plant Volatiles (OIPVs), and they can
65 attract egg parasitoids, but several plant species respond to insect egg deposition by changing
66 leaf surface cues that tend to retain parasitoids on leaves with host eggs (Hilker and Fatouros,
67 2015).

68 In tea ecosystems, various species of insect pests can occur in the same area, resulting in
69 differences in herbivore species, number of feeding individuals, type of feeding, and feeding
70 time. These factors in turn lead to different chemical blends produced by egg-laden or
71 feeding-damaged plants (De Moraes, Mescher, and Tumlinson, 2001; Hare and Sun, 2011),
72 and these chemical cues can be used by parasitoids and predators to find their hosts or prey.
73 Parasitoids must be able to discriminate the different blends of chemicals released from
74 plants infested by host or non-host insects (Erb, Foresti, and Turlings, 2010; Ponzio et al.,
75 2016; Thanikkul, Piyasaengthong, Menezes-Netto, Taylor, and Kainoh, 2017).

76 In this study, we focused on species-specific elicitors that induce tea leaves to release
77 chemicals that arrest the egg-larval parasitoid *A. reticulata*. We predicted that non-host
78 insects would have a neutral or negative influence on host searching of this parasitoid. Two
79 species of non-host pest insects, i.e., the Oriental tea tortrix, *Homona magnanima* Diakonoff
80 (Lepidoptera: Tortricidae), and the Asian corn borer, *Ostrinia furnacalis* (Guenée)
81 (Lepidoptera: Crambidae), were used for experimental treatments following the method of
82 Piyasaengthong et al. (2016b). *Homona magnanima* is an economically important pest that
83 often occurs together with *A. honmai* on tea plants in Japan, and *O. furnacalis* is the most
84 serious insect pest of maize, *Zea mays* L., in many countries, including Japan and Thailand
85 (Patanakamjorn, 1975; Huang et al., 1998). Thus, we tested whether *A. reticulata* females can
86 discriminate changes on the adaxial surface of tea leaves after treatment with homogenized
87 reproductive tissues of a non-host insect (*H. magnanima* or *O. furnacalis*) compared with
88 those on untreated leaf (control). In our previous study, the female parasitoids stayed longer

89 on tea leaves treated with homogenized reproductive tissues of the host insect (*A. honmai*)
90 than that on untreated leaves (Piyasaengthong et al., 2016b).

91

92 **Materials and Methods**

93 *Host insect and parasitoid rearing*

94 We reared *Adoxophyes honmai* as described by Tamaki (1966) and *A. reticulata* as described
95 by Kainoh (1988). Both hosts and parasitoids were obtained from colonies that were reared
96 under laboratory conditions ($25 \pm 1^\circ\text{C}$, $60 \pm 10\%$ RH, 16L:8D photoperiod) on an artificial
97 diet containing tea leaf powder. Other rearing details were described by Piyasaengthong,
98 Sato, Kinoshita, and Kainoh (2016a).

99

100 *Non-host insect rearing*

101 *Homona magnanima* was obtained from the Institute of Fruit Tree and Tea Science, NARO
102 (Shimada, Shizuoka, Japan), and reared on a mulberry leaf-based artificial diet (Insecta LFS,
103 Nosan Corp., Yokohama, Japan). *Ostrinia furnacalis* was obtained from the Japan Plant
104 Protection Association's Ibaraki Research Institute (Ushiku, Ibaraki, Japan) and reared on an
105 artificial diet (Silk Mate 2M, Nosan Corp., Yokohama, Japan). Individuals of both species
106 were sexed at the pupal stage and reared separately until adult emergence. Male and female
107 *H. magnanima* moths were kept in rearing containers (25cm × 18cm × 8 cm) containing moist
108 cotton and a few pieces of folded wax paper for oviposition. *Ostrinia furnacalis* was kept in a
109 cylindrical chamber (13 cm in diameter, 33 cm long); the inside of the chamber was covered
110 with wax paper for oviposition, and 10% sugar solution was provided as food. Both colonies
111 were kept in a room under controlled conditions at $25 \pm 1^\circ\text{C}$ and $60 \pm 20\%$ RH with a
112 16L:8D photoperiod.

113

114 *Plant rearing*

115 Ten-year-old potted tea plants were kept in a greenhouse at 14L ($25 \pm 1^\circ\text{C}$):10D ($20 \pm 1^\circ\text{C}$)
116 and 40–60% RH and were watered every 2 days. Five plants treated with reproductive
117 homogenates were kept separate from 5 untreated plants. Old leaves used for bioassays were
118 selected by their colour (see supplementary Figure 1 from Piyasaengthong et al., 2016a) and
119 position (the stem side of a branch).

120

121 *Reproductive system treatment of non-host insects*

122 To homogenize the reproductive system of non-host insects, we dissected a mated female (3
123 to 4-day-old) of either species and placed the ovaries, accessory glands, bursa copulatrix, and
124 spermatheca from an abdomen in a 1.5-ml microtube with 10 μl of lepidopteran Ringer's
125 solution (Blum, 1985) and homogenized them with a plastic grinding rod (Fig. 1A).

126 However, the abdomen of *H. magnanima* is larger than that of *O. furnacalis* which is similar
127 to *A. honmai*, therefore, only 27 μl of homogenate was needed in the treatment as our
128 previous experiment with *A. honmai* (Piyasaengthong et al., 2016b) to treat a proper amount
129 of the homogenate on a tea leaf. The homogenate was placed on Parafilm[®] and attached to the
130 abaxial side of a leaf of a potted tea plant for 24 h. The edges of the Parafilm[®] were secured
131 by cellophane tape (Fig. 1B). For untreated leaves in all treatments, Ringer's solution (27 μl)
132 alone was applied. All details above were prepared as one replicate of our experiments (1 set
133 of treated and untreated leaves). Five replicates were performed for each species of non-host
134 insects.

135

136 *Contact bioassays of species-specific elicitor of induction*

137 To test whether the induction of tea leaves surface chemical is species-specific, we performed
138 two-choice tests using a leaf treated with homogenized reproductive system of a mated

139 female of one species and an untreated leaf from a different plant. All test leaves were
140 plucked from the plant, the Parafilm[®] and tape were removed. The leaves were carefully
141 washed under tap water and then with distilled water to remove all deposits (Fig. 1C). A set
142 of treated and untreated leaves was attached (~2 cm apart) to the bottom of a plastic Petri dish
143 (14 cm diameter, 2 cm deep) with double-sided tape on the abaxial side (Fig. 1D). A virgin
144 female parasitoid (2 to 4-day-old) with no previous oviposition experience was released at the
145 centre of the Petri dish, which was covered with a lid after the wasp was released. We used
146 virgin females, since there was no apparent difference in fecundity and response to host
147 kairomone between mated and unmated females (Honda, Kainoh, and Honda, 1999). The
148 time that each parasitoid spent searching on both leaves was recorded by an event-recording
149 software (The Observer v. 5.0; www.noldus.com) for 10 min (N=25 wasps/treatment).

150 To confirm whether the induction phenomenon was specific to moth species, we analysed
151 and compared the residence times of *A. reticulata* females on treated and untreated leaves. In
152 the data analyses, we used a linear mixed-model to consider the effect of pairs of treated and
153 untreated leaves as a random factor (“lmer” in the packages *lme4*; Bates, Maechler, Bolker,
154 and Walker, 2015), because we reused sets of treated and untreated leaves five times (five
155 parasitoids per set of leaves). We used an offset with the log-transformed total residence time
156 in the model to consider the activity of individual parasitoids (Faraway, 2006; Crawley,
157 2007). Fixed factors in the model were treatment (treated or untreated), type of homogenate
158 (reproductive tissues from *H. magnanima* or *O. furnacalis*), and their interaction. All
159 statistical analyses were performed with R v. 3.1.3 software (R Core Team, 2015).

160

161 **Results**

162 Because the effect of the interaction between treatment and type of homogenate was
163 significant (likelihood ratio test; $\chi^2=7.2637$, $df= 1$, $P= 0.007$), we analysed the data for each

164 type of homogenate. The residence time of parasitoids on leaves treated with *H. magnanima*
165 homogenate was 25 ± 7 s (mean \pm SE). Although this was longer than that spent on untreated
166 leaves (19 ± 4 s), the difference was not significant (likelihood ratio test; $\chi^2 = 0.7993$, $df = 1$,
167 $P = 0.371$). In contrast, the residence time on leaves treated with the *O. furnacalis*
168 homogenate (20 ± 4 s) was significantly shorter than that spent on untreated leaves (50 ± 11
169 s; $\chi^2 = 6.9205$, $df = 1$, $P = 0.009$; Fig. 2).

170

171 **Discussion**

172 Treatments of different moth species affected the residence time that parasitoids spent on the
173 tea leaves. The residence time of *A. reticulata* females on leaves treated with homogenates of
174 *H. magnanima*, a herbivore of tea, was not significantly different from that on untreated
175 leaves. By contrast, the female parasitoids stayed longer on untreated leaves than on leaves
176 treated with homogenates of *O. furnacalis*, which feeds on maize.

177 Tritrophic interaction among *C. sinensis*, the herbivorous insect *A. honmai*, and its
178 parasitoid *A. reticulata* is interesting because tea plant surface chemicals can be induced by
179 egg deposition of *A. honmai* without any obvious damage to the leaf surface (Deshpande and
180 Kainoh, 2012; Piyasaengthong et al., 2016b). A similar phenomenon also occurs in the
181 tritrophic interaction among Brussels sprout (*Brassica oleracea* var. *gemmifera*), the large
182 cabbage white butterfly (*Pieris brassicae* L.), and the egg parasitoids *Trichogramma*
183 *brassicae* Bezdenko (Fatouros et al., 2005, 2009; Pashalidou, Huigens, Dicke, and Fatouros,
184 2010) and *Trichogramma evanescens* Westwood (Fatouros, Bukovinszkine'Kiss, Dicke, and
185 Hilker, 2007). If tea plants can be induced to change the surface chemicals without
186 experiencing physical damage, this suggests the presence of an elicitor from the female
187 reproductive organs at the time of oviposition.

188 Many studies have reported that chemicals in the female reproductive system, associated
189 with egg deposition, act as elicitors (e.g., Meiners and Hilker, 2000; Hilker, Kobs, Varama,
190 and Schrank, 2002; Fatouros et al., 2009; Blenn et al., 2012), which confirms our previous
191 findings (Piyasaengthong et al., 2016b). In that study, the residence time of female *A.*
192 *reticulata* on tea leaves treated with homogenized reproductive tissues from virgin or mated
193 *A. honmai* females was significantly longer than that on untreated leaves at 24 h after
194 treatment (Fig. 2). No such induction occurred when leaves were treated with male
195 abdominal contents. Therefore, the elicitor in this system appears to be female-specific.

196 In complex habitats in the field, many insect species share foraging sites in the same area,
197 thus influencing the host-searching behaviour of natural enemies. Different insect species
198 tend to produce different types of elicitors, e.g., β -glucosidase (Mattiacci, Dicke, and
199 Posthumus, 1995), volicitin (Alborn et al., 1997) and inceptin (Schmelz et al., 2006). These
200 different elicitors from each herbivorous insect species result in different responses of each
201 female parasitoid to the leaves. The elicitor from egg stage should be different from those
202 cases in larval stages, in which saliva is a major source of elicitor. In the case of egg-
203 associated secretions, those from female accessory reproductive gland (ARG) or oviductus
204 communis elicit defensive responses from plants (Hilker and Fatouros, 2015). Chemical
205 analysis of the elicitor from *A. honmai* egg or adult abdominal contents will be a future
206 research subject.

207 Our experimental results indicate that the treatments of non-host insects on the tea leaf
208 affects the host searching of *A. reticulata*. The female *A. reticulata* showed a neutral response
209 to tea leaves treated with reproductive homogenate of *H. magnanima*, a non-host insect that
210 also feeds on *C. sinensis*. Another interesting question is why the parasitoid had a negative
211 response to leaves treated with tissues of *O. furnacalis*, a non-host insect that feeds on maize.
212 In our experiment, we used only the homogenate, not natural egg-laying by the moth.

213 Therefore, there appear to be materials in this homogenate that induce a different set of
214 chemicals by leaf tissue, which the parasitoid may recognize as “non-host” chemicals. The
215 production of species-specific leaf surface chemicals by the host plant would be
216 advantageous for parasitoids that are not interested in this insect, because it allows them to
217 recognize that they do not need to spend time searching the leaf. The degree of induction may
218 be related to the taxonomic distance, since *A. honmai* and *H. magnanima* belong to the same
219 family Tortricidae. In order to explain these species-specificity, we need to extend the
220 sample species of host and non-host herbivores.

221 All these ecological issues should be further investigated. For example, it is necessary to
222 determine a critical period of induction (from oviposition to surface chemical change of the
223 leaf) and dose-response of elicitors (treating different doses of hosts and non-host insects),
224 and also identifying species-specific elicitors might help to elucidate the tritrophic
225 interactions at the molecular level (e.g., gene expression patterns of treated versus untreated
226 tea leaves).

227

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234

235 **Author Contribution**

236 NP, YS and YK contributed to the design and implementation of the research, to the analysis
237 of the results and to the writing of the manuscript.

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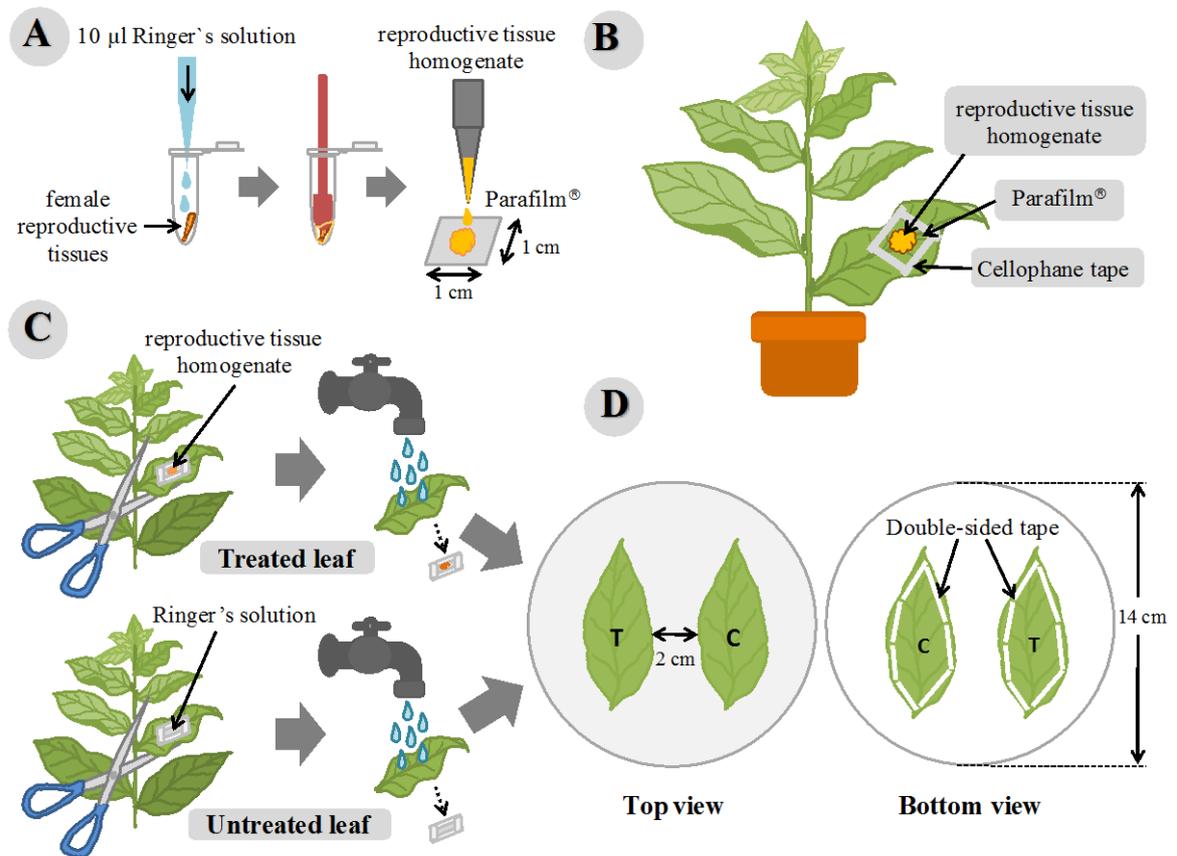
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351 **Fig. 1. (A)** Preparation of homogenized female reproductive tissues before use for treatment

352 on tea leaves. **(B)** Application of homogenate on the under surface of the tea leaf, covered

353 with paraffin film and cellophane tape (for control, only Ringer's solution was applied). **(C)**

354 Preparation of treated and untreated leaves for contact bioassay. **(D)** Top and bottom views of

355 the experimental setup for the contact bioassays (two-choice test) using a Petri dish and a pair

356 of leaves; treated leaf (T) and untreated leaf (C).

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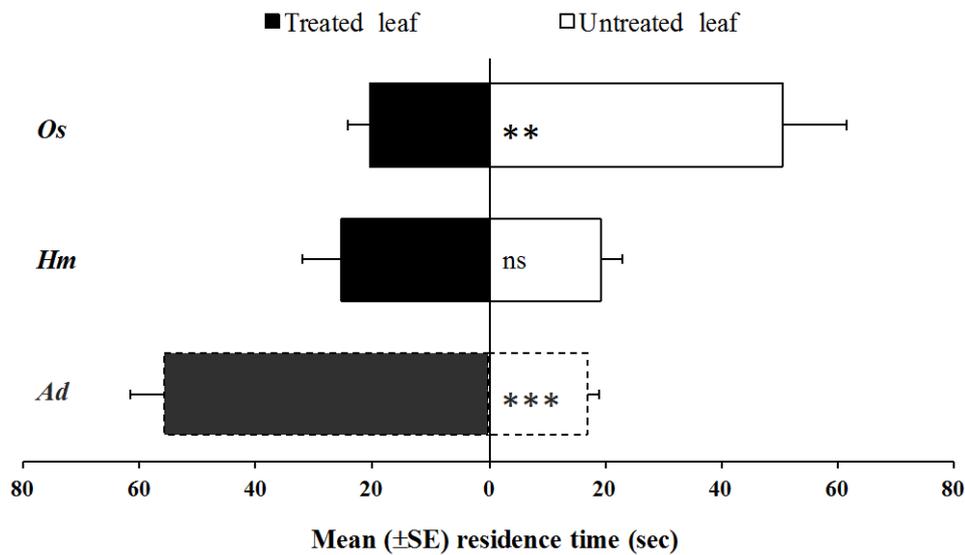
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364 **Fig. 2.** Effects of species-specific elicitor on induction of chemical release by tea leaf. Mean
 365 residence time of *Ascogaster reticulata* parasitoids on treated and untreated leaves in two-
 366 choice contact bioassays are shown. Test leaves were treated with homogenized reproductive
 367 system of mated female *Homona magnanima* (*Hm*), *Ostrinia furnacalis* (*Os*), or *Adoxophyes*
 368 *honmai* (*Ad*; Piyasaengthong et al., 2016b) for 24 h; only Ringer's solution was applied to
 369 untreated leaves. Number of female parasitoids tested per bioassay: *H. magnanima*, $n = 25$;
 370 *O. furnacalis*, $n = 25$; *A. honmai*, $n = 30$. Asterisks indicate a significant difference
 371 (** $P < 0.01$; *** $P < 0.001$) between treated and untreated leaves.

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