

1 **5. Mechanism of β -Aminobutyric Acid-Induced Resistance in Wheat to the Grain**
2 **Aphid, *Sitobion avenae* or**
3 **Dissecting/Deciphering the Mechanism of β -Aminobutyric Acid-Induced**
4 **Resistance in Wheat to the Grain Aphid, *Sitobion avenae***
5 **Short title: β -Aminobutyric Acid-Induced Resistance to *S. avenae***

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45 **Abstract:**

46 The non-protein amino acid β -aminobutyric acid (BABA) could induce plant resistance to a
47 broad spectrum of biotic and abiotic stresses. However, BABA-induced plant resistance to
48 insects is less well-studied, especially its underlying mechanism. In this research, we
49 applied BABA to wheat seedlings and tested its effects on *Sitobion avenae*. When applied
50 as a soil drench, BABA significantly reduced weight of *S. avenae*, whereas foliar spray
51 and seed treatment had no such effects. BABA-mediated suppression of *S. avenae*
52 growth is dose dependent and could last at least for 7 days. The aminobutyric acid
53 concentration in phloem sap of BABA-treated plants accumulated to high levels and
54 increased with BABA concentrations applied. Moreover, after 10 days of treatment, the
55 aminobutyric acid content in BABA-treated plants was still higher than that in control
56 treatment. *S. avenae* could not discriminate artificial diet containing BABA from standard
57 diet, indicating that BABA itself is not a deterrent to this aphid. Also *S. avenae* did not
58 show preference for control plants or BABA-treated plants. Consistent with choice test
59 results, *S. avenae* had similar feeding activities on control and BABA-treated plants,
60 suggesting that BABA did not induce antifeedants in wheat seedlings. In addition,
61 aminobutyric acid concentration in *S. avenae* feeding on BABA-treated plants was
62 significantly higher than those feeding on control plants. *S. avenae* growth rate was
63 reduced on artificial diet containing BABA, indicating direct toxic effects of BABA to this
64 aphid. These results suggest that BABA application could enhance wheat plant resistance
65 to *S. avenae* and the mechanism is possibly due to direct toxicity of high BABA contents in
66 plant phloem.

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90 Introduction

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92 The non-protein amino acid β -aminobutyric acid (BABA) could enhance plant resistance
93 against a broad spectrum of phytopathogens. BABA-induced plant resistance is effective
94 against viruses, bacteria, oomycetes, fungi and phytopathogenic nematodes [1-4]. The
95 mechanism of BABA-induced plant resistance to different pathogen is variable.
96 BABA-induced *Arabidopsis* resistance against the oomycete pathogen *Peronospora*
97 *parasitica* is based on callose deposition and independent of jasmonic acid (JA), salicylic
98 acid (SA) and ethylene signaling pathways, whereas against the bacteria *Pseudomonas*
99 *syringae* is solely dependent of SA and NPR1 [3]. Enhanced resistance of tobacco to
100 tobacco mosaic virus by BABA application was found to be strictly dependent on SA
101 pathway [2]. BABA-mediated grapevine resistance to downy mildew (*Plasmopara viticola*),
102 however, is based on potentiating callose formation and JA signaling [5]. In addition to
103 conferring plant resistance to pathogens, BABA also improves plant tolerance to salt,
104 drought and high temperature, which is associated with accumulation of the abscisic acid
105 [6-8]. *Arabidopsis* mutants that are insensitive to BABA-induced sterility have reduced
106 resistance level to pathogen or tolerance to salt, suggesting that BABA-induced plant
107 resistance to biotic and abiotic stresses has a genetic basis [9].

108 BABA also has been demonstrated to be effective in plant resistance to insects [10-12].
109 Hodge et al. (2005) found that applied BABA as a root drench to six legume plants
110 reduced the performance of the pea aphid, *Acyrtosiphon pisum* (Harris) [10]. This is the
111 first study examined the effects of BABA-mediated plant resistance to insects. When
112 applied to Brassicaceae plants, BABA suppressed the growth of phloem-feeding insects
113 *Myzus persicae* and *Brevicoryne brassicae* as well as chewing insects *Trichoplusia ni* and
114 *Plutella xylostella* [11]. Recently, Tiwari et al. (2013) reported that BABA application could
115 also induce citrus resistance to the Asian citrus psyllid, *Diaphorina citri* Ku wayama, and
116 the authors did not find any direct toxicity of BABA to this insect by leaf-dipping bioassays
117 [12]. Although these studies indicate that BABA could enhance plant resistance to insects,
118 little is known about the underlying mechanism.

119 Plant direct resistance to insects relies largely on metabolites that exert toxic,
120 antinutritive, or repellent effects. Insects could perceive these compounds by
121 chemoreceptors and decide to accept or reject a host [13]. The phloem-feeding insects,
122 like aphids and whiteflies, have evolved specialized mouthparts, the stylets, which
123 penetrate plant epidermis, pass through intercellular tissue and the second wall material,
124 searching for sieve element. During stylet penetration, aphids regularly puncture plant
125 cells and taste cytosolic contents to decide to feed or leave before reaching phloem [14].
126 Once reached to the sieve element, aphids will always eject watery saliva to prevent sieve
127 tube plugging before phloem sap ingestion. The probing behavior of aphids can be
128 monitored by the electrical penetration graph (EPG) technique [15].

129 The English grain aphid, *Sitobion avenae* is a major pest of cereal worldwide [16]. This
130 aphid causes substantial losses of cereal yield by removing photoassimilates and
131 transmitting viruses. Application of chemical pesticides is still the main method to control
132 this aphid; however, chemical control causes negative impacts on agroecosystem and can

133 lead to insect resistance to pesticides [16]. Thus searching for alternative methods to
134 control this aphid is of great significance.

135 Understanding the mechanism of BABA-induced plant resistance to insects will shed
136 light on new methods of pest control. In this study we examined the effects of BABA and
137 its isomers α -aminobutyric acid (AABA) and γ -aminobutyric acid (GABA) application to
138 wheat on performance of the *S. avenae*. The durability of BABA-induced resistance was
139 also investigated. Host preference and feeding behavior of *S. avenae* were studied to
140 localized possible resistance factors. We found that BABA accumulated to high
141 concentration in BABA-treated wheat phloem sap, so we use aphid artificial diet to test the
142 direct toxicity of BABA to *S. avenae*.

143

144 **Materials and Methods**

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146 **Plants and Insects**

147 Seeds of winter wheat, *Triticum aestivum* (var. XiNong 979), were germinated at room
148 temperature ($25 \pm 1^\circ\text{C}$) for 2 days. Seedlings with similar size were then transplanted to
149 pots (250 mL) containing a 5:1 mixture of peat moss (Pindstrup Mosebrug A/S,
150 Ryomgaard, Denmark) and perlite. Seedlings were grown singly in pot unless otherwise
151 indicated. Plants were cultivated in a walk-in growth chamber at $24 \pm 1^\circ\text{C}$, $60 \pm 5\%$ relative
152 humidity and a 14:10 hour light/dark regime. The plants were watered as necessary. The
153 grain aphid, *S. avenae*, originally collected from a winter wheat field in Yangling, China,
154 was reared on wheat seedlings (var. XiNong 979) in the same growth chamber.

155

156 ***S. avenae* Performance on Wheat Seedlings**

157 Seven-day-old wheat seedlings were treated as indicated and then 2-3 apterous
158 *S.avenae* adults were introduced to the first leaf of each seedling. The seedlings were
159 caged by transparent plastic cages (8 cm in diameter and 30 cm in height) secured with
160 nylon mesh at the top. One day later, the adults were removed, leaving 5-6 nymphs on the
161 first leaf. After another 7 days, aphids on the first leaves were collected and weighed on a
162 microbalance (resolution 0.001 mg; Sartorius MSA 3.6P-000-DM, Gottingen, Germany).
163 To assess the effects of BABA treatment on plant growth, the fresh shoot weights of wheat
164 seedlings were weighed after aphid collection. Weights of aphids feeding on wheat
165 seedlings soil drenched with MilliQ water, different concentrations of BABA, and 50 mM
166 GABA were analyzed using one-way analysis of variance (one-way ANOVA) following by
167 Tukey's HSD (Honestly Significant Difference) test. Mean aphids weights on plants soil
168 drenched with 50 mM AABA, sprayed with BABA, or seed treatment with BABA were
169 compared with respective control by Student's *t*-test. Wheat seedlings weights were
170 analyzed using one-way ANOVA following by Tukey's HSD test. All statistical analyses in
171 this research were conducted using IBM SPSS Statistics package (version 19.0; SPSS
172 Inc., Chicago, IL, USA).

173

174 ***S. avenae* Settling Choice Tests**

175 Seven-day-old wheat seedlings were soil drenched with 20 mL MilliQ water (control) or
176 25 mM BABA. One day after treatment, one control and one BABA-treated seedling were

177 put in a transparent plastic cage (26 cm height × 15 cm length × 14 cm width) secured
178 with nylon mesh at the top. Then 15 alate adults of *S. avenae* were introduced to each
179 cage and the numbers of *S. avenae* on each seedling were recorded 4, 8, 24 and 48 h
180 after the start of the experiment. This experiment was carried out in a completely dark
181 room and 8 replicates were conducted. Numbers of aphids on control and BABA-treated
182 plants at each time point were compared by paired *t*-test.

183 To determine whether BABA itself has direct antifeedant effects, we tested *S. avenae*
184 preference to artificial diet containing BABA. The composition of standard artificial diet
185 was based on Auclair (1965) [17] with modifications (Table S1). After sterile filtering
186 through Millex GP syringe filters (hydrophilic polyethersulfone membrane, pore size 0.22
187 µm; Ireland), the artificial diets were stored at -70°C until use. The Petri dish lid (7 mm in
188 height and 37 mm in diameter) was covered with a layer of stretched Parafilm® M
189 (Chicago, IL). Forty µL of MilliQ water (H₂O), artificial diet (Control), and artificial diet
190 containing 50 mM BABA were put on the Parafilm® M separately. The test solutions were
191 then covered with another layer of Parafilm® M. We made a hole (5 mm in diameter) on
192 the back of each lid and introduced twenty one 3rd-5th instar aphids through this hole.
193 The numbers of aphids feeding on each test solution were recorded after 6, 12, 24, and 48
194 h. Fourteen replicates were performed. Percentages of aphids on each test solution within
195 each recording time point were analyzed with one-way ANOVA and means were
196 compared using Tukey's HSD test.

197

198 *S. avenae* Feeding Behavior

199 Aphid feeding activities on control and BABA-treated plant seedlings were recorded by
200 the Giga-8 direct-current electrical penetration graph (DC-EPG) system [18]. The detail of
201 this experiment can be found in our previous work [19]. Wheat seedlings were soil
202 drenched with 20 mL MilliQ water (Control) or 25 mM BABA two days before EPG
203 recording. Each apterous adult and wheat seedling was used once. Data were recorded
204 by the Stylet+d software and analyzed with the Stylet+ software. The stylet pathway
205 waveforms were distinguished according to Tjallingii (1978) [15]. EPG parameters were
206 calculated using the Excel workbook for automatic parameter calculation of EPG data 4.3
207 [20]. Because the EPG data were not normally distributed, paired comparison of means of
208 control treatment and BABA treatment was done by non-parametric Mann–Whitney
209 *U*-test.

210

211 Extraction and Analysis of Amino Acids

212 We used EDTA-facilitated exudation method to collect phloem sap of wheat seedling
213 leaves for free amino acids analysis. Wheat seedlings were soil drenched as described 3
214 days before sample collection. The first leaf of each seedling was cut 1 cm above the
215 leaf/stem junction and immediately put in 1.5 mL EP tube containing 600 µL EDTA solution
216 (pH = 7.1). Two leaves were put in a tube and regarded as one replicate. Then these cut
217 leaves were put in a completely dark growth chamber at 25°C 100% humidity for 3 hours.
218 The samples were stored at -70°C until analysis.

219 Newly born *S. avenae* nymphs feeding on control and BABA treated plants for 7 days
220 were collected and weighed. Free amino acids in aphids were extracted with 50% ethanol

221 containing 0.1 M HCl. Mean concentrations of each amino acid from different treatments
222 were compared by Student's *t*-test.

223 The free amino acids extracted from plants and aphids were analyzed by LTQ XL™
224 linear ion trap mass spectrometer (Thermo Scientific, USA). Liquid chromatography
225 separations were carried out with XTerra MS C18 Column (125Å pore size, 5 µm, 150 mm
226 × 4.6 mm; Waters Corp., USA). Amino acids elution was performed applying a three-step
227 gradient: A 100% for 7 min, 0-100% B linear for 2 min, 100% B for another 5 min, 0-100%
228 A linear for 1 min, holding the system at 100% A for 5 min. Mobile phase A was a aqueous
229 solution containing 5% acetonitrile and 0.1% formic acid; mobile phase B was 100%
230 acetonitrile. The flow rate was 0.6 mL/min. The mass spectrometer worked in the positive
231 electrospray ionization (ESI) mode. Nitrogen was used as the sheath gas (50.0 arbitrary
232 units) and auxiliary gas (8.0 arbitrary units). The spray voltage was set at 4.5 kV and the
233 ion transfer capillary temperature was 320°C. The amino acids were scanned and
234 fragmented using data dependent MS/MS. Masses of precursor and product ions and
235 collision energy for each amino acid were as described in Table S1. Data were acquired
236 and processed using Xcalibur 2.1 software (Thermo Scientific). Quantification was
237 achieved by external standard amino acid mixture of known concentrations (AA-S-18,
238 Sigma). For our method can not detect glycine, our data do not include glycine.

239

240 Time Course Assay

241 To investigate the durability of the effects of BABA treatment on *S. avenae* performance
242 and phloem sap amino acids composition, we conducted a time course experiment.
243 Seven-day-old wheat seedlings were soil drenched with 20 mL MilliQ water or 25 mM
244 BABA, and aphid performance assays started 0, 7, and 14 days after treatment; phloem
245 sap were collected 3, 10, and 17 days after treatment for amino acids analysis. Aphid
246 weights and aminobutyric acid concentrations of different treatments within each sample
247 day were analyzed by Student's *t*-test respectively.

248

249 Peroxidase Assays

250 Peroxidase (POD) is involved in some forms of plant resistance to insects [21]. The
251 first leaves of control and BABA-treated plants were collected 2, 4, and 6 days after
252 treatment, extracted and analyzed as described previously [19]. POD activities of control
253 and BABA-treated plants within each sample day were compared by Student's *t*-test. In
254 another experiment, plants were divided to following four treatments: (1) control plants
255 (soil drenched with water); (2) control plants infested with fifteen 3rd instar-adult *S.*
256 *avenae*; (3) BABA-treated plants (soil drenched with 25 mM BABA); (4) BABA-treated
257 plants infested with fifteen 3rd-adult *S. avenae*. After 2, and 4 days of treatment, the first
258 leaves were collected and analyzed as described. Mean activities of POD among
259 treatments on each day were analyzed by one-way ANOVA, with Tukey's HSD post-hoc
260 test.

261

262 Artificial Diet Assays

263 We tested the direct impacts of BABA on performance of *S. avenae* by measuring *S.*
264 *avenae* nymph growth on standard artificial diet and artificial diet containing 50 mM BABA,

265 AABA, or GABA. We use AABA and GABA in artificial diet to exclude the possibility that
266 the imbalance of artificial diet have negative impacts on aphid growth. Thirty-five μL of
267 artificial diet was confined between two layers of stretched Parafilm[®] M on plastic cylinder
268 (1 cm in height and 1cm in diameter) and the artificial diet was replaced every two days.
269 Five one-day-old nymphs produced by apterous *S. avenae* were transferred to one tube
270 containing one kind of artificial diets and regarded as one replicate. The weight of
271 individual nymph was weighed on the MSA 3.6P-000-DM microbalance after feeding on
272 artificial diet for 4 days. We also tested aphid performance on artificial diet containing 50
273 mM and 100 mM BABA with similar manner, but the one day old nymphs used were
274 produced by *S. avenae* collected from wheat seedlings (var. XiNong 979) in greenhouse.
275 Weights of aphids were analyzed with one-way ANOVA following by Tukey's HSD test.

276

277 **Results**

278 *S. avenae* Performance

279 Compared with control treatment, BABA applied as a soil drench significantly reduced
280 weights of *S. avenae* on wheat seedlings and the effects increased with BABA
281 concentration (Fig. 1A). In contrast, soil drench with GABA (Fig. 1A) and AABA (Fig. 1B),
282 spray with BABA (Fig. 1C), or seed treatment with BABA (Fig. 1D) had no negative
283 impacts on *S. avenae* weights.

284

285 *S. avenae* Preference

286 BABA-treated wheat plants were not attractive or repellent to *S. avenae* (Fig. 2A). *S.*
287 *avenae* preferred artificial diets than MilliQ water, but showed no preference between
288 standard artificial diet and artificial diet containing 50 mM BABA (Fig. 2B).

289

290 Free Amino Acids Composition of Phloem Sap

291 The relative concentration of aminobutyric acid in phloem sap of BABA-treated wheat
292 plants was higher than that in control plants (Fig. 3). Our method can not discriminate
293 BABA from its isomers, the excess amount of aminobutyric acid in BABA-treated plants
294 was assumed to be BABA. Because wheat plants could produce GABA not BABA, we
295 consider the aminobutyric acid in control plants was GABA. Soil drenched with AABA did
296 not influence aminobutyric acids concentration in wheat phloem, while GABA only slightly
297 increased aminobutyric acids levels.

298 Except BABA, the relative concentration of serine in BABA-treated wheat phloem sap
299 was also significantly less than that in control plants (Student's *t*-test, $t = 2.78$, $df = 10$, $P =$
300 0.02 ; Fig. S2), while other amino acids levels between treatments were similar (Fig. S2).

301

302 Free Amino Acids Composition of *S. avenae*

303 The relative concentration of aminobutyric acid in *S. avenae* feeding on BABA-treated
304 plants was significantly higher than those feeding on control plants (Student's *t*-test, $t =$
305 8.84 , $df = 6$, $P < 0.001$; Fig. 4). The high levels aminobutyric acid in *S. avenae* should be
306 BABA from phloem sap of BABA-treated plants. The relative levels of alanine ($P = 0.052$)
307 and threonine ($P = 0.073$) in aphids feeding on BABA-treated plants were slightly lower
308 than those feeding on control plants (Fig. 4). The other amino acids composition pattern

309 was similar.

310

311 Durability of BABA Treatment

312 After 7 days of BABA treatment, wheat seedlings still could reduce *S. avenae* growth
313 (Student's *t*-test, $t = 3.10$, $df = 56$, $P < 0.01$; Fig. 5A), which is positively correlated with
314 aminobutyric acid levels in plant phloem sap (Fig. 5B). The relative aminobutyric acid
315 concentration in BABA-treated plants was higher than that in control plants 3 days and 10
316 days after treatment, but not significant after 17 days (Fig. 5B).

317

318 Feeding Activities of *S. avenae*

319 The feeding behavior of *S. avenae* on BABA-treated and control plants was similar
320 revealed by EPG technique (Table 1). The number of probes, total duration of probing,
321 and total duration of phloem feeding (E2) were not significantly different, suggesting that
322 BABA treatment did not induce physical barriers or chemical deterrents in wheat seedlings
323 against *S. avenae* (Table 1).

324

325 POD Activities

326 There were no difference of POD activities between control and BABA-treated plants
327 (Fig. S3A). Also BABA treatment did not increase POD activities of aphid infested wheat
328 seedlings compared with aphid infested control plants (Fig. S3B).

329

330 Direct Toxicity of BABA on *S. avenae*

331 *S. avenae* feeding for 4 days on artificial diets containing BABA or GABA had lower
332 weight than those feeding on standard artificial diet; whereas those feeding on artificial
333 diet containing AABA had no influence on aphid weight (one-way ANOVA, $F = 11.92$, $df =$
334 $3, 35$, $P < 0.001$; Fig. 6A). *S. avenae* feeding on artificial diet containing BABA had only 55%
335 weight of those feeding on standard artificial diet (Fig. 6A). *S. avenae* feeding for 3 days
336 on standard artificial diet had higher weight than those feeding on artificial diets containing
337 50 mM or 100 mM BABA (one-way ANOVA, $F = 11.33$, $df = 2, 49$, $P < 0.001$; Fig. 6B). The
338 mortality of *S. avenae* feeding on all artificial diets was similar (data not shown).

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342 Discussion

343 Although there have been numerous studies examined the effects and mechanisms of
344 BABA-mediated plant resistance to plant pathogens, few papers investigated the role of
345 BABA in plant resistance to insects [4,9]. The already published papers suggest that
346 BABA application could suppress performance of some aphids, caterpillars, and psyllid
347 [10-12]. Our results demonstrated that BABA applied as a root drench could significantly
348 reduce weight of *S. avenae* and the effects was dose dependent. We found high
349 concentration of BABA in treated plant phloem sap, and the BABA level was still higher
350 than that in control phloem sap after 10 days, suggesting that wheat could absorb BABA
351 by roots and metabolize or decompose BABA slowly. The BABA-mediated suppression of
352 aphid growth could last at least for 7 days, which is correlated with BABA contents in

353 phloem sap. However, we did not find any influence of BABA application on *S. avenae*
354 host preference or feeding behavior, indicating that BABA did not induce any repellents in
355 wheat seedlings. It was assumed that resistance to insects induced by BABA is not based
356 on its direct toxicity [10,12]. In contrast, we found that *S. avenae* feeding on artificial diet
357 containing BABA had reduced weight, suggesting that BABA has direct toxic effects on *S.*
358 *avenae*.

359 Our results demonstrated that only when drenched into the soil, BABA could reduce *S.*
360 *avenae* growth on wheat. And BABA-mediated wheat resistance to *S. avenae* is positively
361 correlated with BABA concentration in wheat phloem sap. By using ¹⁴C-labeled BABA,
362 Cohen and Gisi (1994) found that only a small proportion of sprayed BABA is taken up by
363 plant leaves [22]. Foliar spray and seed treatment with BABA may result in relative lower
364 BABA concentration in wheat phloem and thus have no effects on aphid performance.
365 Root drench with BABA rendered wheat plants a durable resistance to *S. avenae*, which
366 corresponds to high BABA concentration in wheat phloem. Our time course experiment
367 showed that BABA concentration in treated plants decreased with time, which suggests
368 that BABA could be metabolized by wheat plants slowly.

369 *S. avenae* cannot discriminate artificial diet containing BABA from standard artificial diet,
370 implying that *S. avenae* may not have corresponding receptors. Because BABA is rarely
371 found in plants, aphids have few chances to encounter this compound in nature and do
372 not evolve the ability to perceive BABA [23]. The EPG data indicate that *S. avenae*
373 exhibited similar feeding activities on control and BABA-treated plants, which is consistent
374 with host preference results. BABA-induced plant resistance to pathogens is mainly
375 through callose formation and SA, ABA signaling pathway [3,9,24]. BABA induced callose
376 deposition at sites of pathogen penetration; thus preventing spread of pathogen [3,24].
377 Callose is also involved in plant phloem sealing mechanisms, which could confer plant
378 resistance to aphids [25,26]. Aphids inject watery saliva to prevent plugging and sealing of
379 sieve plates [26]. This ejection of saliva could be detected by the EPG as E1 salivation.
380 However, total or mean duration of E1 on control and BABA-treated plants was not
381 significantly different. And aphids had similar total as well as mean duration of phloem sap
382 ingestion. Therefore, BABA-mediated wheat resistance to *S. avenae* is not possibly based
383 on callose induced phloem occlusion. JA is the most important cellular signal in plant
384 immunity to most insect herbivores [27]. Application of JA and its derivatives methyl
385 jasmonate could increase plant secondary metabolites and resistance to a broad
386 spectrum of insects [27,28]. Our previous work showed that both methyl jasmonate and
387 SA treatment deterred *S. avenae* preference and feeding activities, but had no effects on
388 aphid performance [19]. These rule out the possible involvement of JA and SA signaling
389 transduction pathways in the BABA-mediated wheat resistance to *S. avenae*.

390 It has been believed that BABA-mediated plant resistance to pathogens and insects is
391 not based on its direct toxicity [10,12,23]. Recently, however, Šašek et al. (2012) found
392 BABA had direct antifungal activity against *Leptosphaeria maculans* and the effect was
393 comparable with the fungicide tebuconazole [29]. Our study showed that both BABA and
394 GABA had direct toxicity to *S. avenae*, when added to artificial diet. GABA is the major
395 inhibitory neurotransmitter in the vertebrates and invertebrates nervous system [30,31].
396 Synthetic diet containing GABA reduced growth and survival of oblique-banded leaf roller

397 larvae [32]. GABA possibly reduced *S. avenae* performance by disturbing aphid nervous
398 system [31]. However, the mechanism of BABA direct suppression of aphid growth is still
399 unknown. In contrast to our findings, Hodge et al. (2005) did not find direct toxicity of
400 BABA to the pea aphid *Acyrtosiphon pisum* and Tiwari (2013) reported that BABA had no
401 influence on Asian citrus psyllid survival [10,12]. It is possible that their application
402 methods only lead to low accumulation of BABA in insects, which is not high enough to
403 reduce insects' fitness. Our data showed that the aminobutyric acid concentration in *S.*
404 *avenae* feeding on wheat plant treated with BABA was 5.8 times higher than that on
405 control plants. The excess aminobutyric acid in *S. avenae* is assumed to be BABA.
406 Another possibility is that the direct toxicity of BABA to insects is species-specific.

407 BABA treatment could alter amino acids balance in *Arabidopsis*, and L-glutamine could
408 inhibit BABA-induced resistance to thermotolerance and a bacterial pathogen [33,34]. We
409 found that BABA-treated wheat phloem accumulated lower serine than water-treated
410 control. These findings imply that BABA possibly involves in plant amino acids metabolite.
411 *S. avenae* feeding on BABA treated wheat seedlings had lower alanine and threonine in
412 their bodies. The lower alanine concentration in *S. avenae* feeding on BABA-treated
413 plants may not be due to alanine difference in plant phloem, because BABA-treated wheat
414 had more alanine. Therefore, this suggests that BABA could also change aphid amino
415 acids metabolite by unknown mechanism. Furthermore, glycine is an important
416 inhibitory neurotransmitter in the central nervous system, while BABA is a partial agonist
417 at the glycine receptor [35]. Low concentrations of BABA competitively inhibits glycine
418 responses, whereas higher concentrations elicits a significant membrane current [35].
419 Therefore, BABA possibly exerts its direct effects to *S. avenae* by causing aphid
420 neurological disorders.

421 BABA treatment did not influence aphid host preference and feeding activities, but
422 resulted in lowered aphid weight and durable high concentration of BABA in wheat phloem
423 sap. Using artificial diet, we clearly showed that BABA had direct toxic effects to *S. avenae*.
424 These findings expand our knowledge of BABA-mediated plant resistance to insects.
425 Further research is needed to investigate whether BABA has direct toxicity to other insects
426 as well as non-target organisms.

427

428

429 **Acknowledgments**

430

431 **Author Contributions**

432 Conceived and designed the experiments: HHC TXL. Performed the
433 experiments: HHC HZ ZFZ XXW. Analyzed the data: HHC MZ ZFZ.
434 Contributed reagents/materials/analysis tools: ZM YZ XXW SSG. Wrote the
435 paper: HHC TXL.

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438 **References**

439

440 1. Oka Y, Cohen Y, Spiegel Y (1999) Local and systemic induced resistance to the

- 441 root-knot nematode in tomato by DL- β -amino-n-butyric acid. *Phytopathology* 89:
442 1138-1143.
- 443 2. Siegrist J, Orober M, Buchenauer H (2000) β -Aminobutyric acid-mediated
444 enhancement of resistance in tobacco to tobacco mosaic virus depends on the
445 accumulation of salicylic acid. *Physiol Mol Plant Pathol* 56: 95-106.
- 446 3. Zimmerli L, Jakab G, Metraux JP, Mauch-Mani B (2000) Potentiation of
447 pathogen-specific defense mechanisms in *Arabidopsis* by β -aminobutyric acid.
448 *Proc Natl Acad Sci U S A* 97: 12920-12925.
- 449 4. Justyna P-G, Ewa K (2013) Induction of resistance against pathogens by
450 β -aminobutyric acid. *Acta Physiologiae Plantarum* 35: 1735-1748.
- 451 5. Hamiduzzaman MM, Jakab G, Barnavon L, Neuhaus J-M, Mauch-Mani B (2005)
452 β -Aminobutyric acid-induced resistance against downy mildew in grapevine acts
453 through the potentiation of callose formation and jasmonic acid signaling. *Mol*
454 *Plant-Microbe Interact* 18: 819-829.
- 455 6. Jakab G, Ton J, Flors V, Zimmerli L, Metraux JP, et al. (2005) Enhancing *Arabidopsis*
456 salt and drought stress tolerance by chemical priming for its abscisic acid
457 responses. *Plant Physiol* 139: 267-274.
- 458 7. Zimmerli L, Hou B-H, Tsai C-H, Jakab G, Mauch-Mani B, et al. (2008) The xenobiotic
459 β -aminobutyric acid enhances *Arabidopsis* thermotolerance. *The Plant Journal* 53:
460 144-156.
- 461 8. Du Y-L, Wang Z-Y, Fan J-W, Turner NC, Wang T, et al. (2012) β -Aminobutyric acid
462 increases abscisic acid accumulation and desiccation tolerance and decreases
463 water use but fails to improve grain yield in two spring wheat cultivars under soil
464 drying. *J Exp Bot* 63: 4849-4860.
- 465 9. Ton J, Jakab G, Toquin V, Flors V, Iavicoli A, et al. (2005) Dissecting the
466 beta-aminobutyric acid-induced priming phenomenon in *Arabidopsis*. *Plant Cell*
467 17: 987-999.
- 468 10. Hodge S, Thompson GA, Powell G (2005) Application of DL- β -aminobutyric acid
469 (BABA) as a root drench to legumes inhibits the growth and reproduction of the
470 pea aphid *Acyrtosiphon pisum* (Hemiptera : Aphididae). *Bull Entomol Res* 95:
471 449-455.
- 472 11. Hodge S, Pope TW, Holaschke M, Powell G (2006) The effect of β -aminobutyric acid
473 on the growth of herbivorous insects feeding on Brassicaceae. *Ann Appl Biol* 148:
474 223-229.
- 475 12. Tiwari S, Meyer WL, Stelinski LL (2013) Induced resistance against the Asian citrus
476 psyllid, *Diaphorina citri*, by β -aminobutyric acid in citrus. *Bull Entomol Res* 103:
477 592-600.
- 478 13. Mithofer A, Boland W (2012) Plant defense against herbivores: chemical aspects.
479 *Annu Rev Plant Biol* 63: 431-450.
- 480 14. Powell G, Tosh CR, Hardie J (2006) Host plant selection by aphids: behavioral,
481 evolutionary, and applied perspectives. *Annu Rev Entomol* 51: 309-330.
- 482 15. Tjallingii WF (1978) Electronic recording of penetration behaviour by aphids. *Entomol*
483 *Exp Appl* 24: 721-730.
- 484 16. Emden HFv, Harrington R (2007) *Aphids as Crop Pests*. CABI, Wallingford, United

- 485 Kingdom: 1-717.
- 486 17. Auclair JL (1965) Feeding and nutrition of the pea aphid, *Acyrtosiphon pisum*
487 (Homoptera: Aphidae), on chemically defined diets of various pH and nutrient
488 levels. *Ann Entomol Soc Am* 58: 855-875.
- 489 18. Prado E, Tjallingii WF (1994) Aphid activities during sieve element punctures. *Entomol*
490 *Exp Appl* 72: 157-165.
- 491 19. Cao H-H, Wang S-H, Liu T-X (2013) Jasmonate and salicylate induced defenses in
492 wheat affect host preference and probing behavior but not performance of the
493 grain aphid, *Sitobion avenae*. *Insect Sci*: (in press).
- 494 20. Sarria E, Cid M, Garzo E, Fereres A (2009) Excel Workbook for automatic parameter
495 calculation of EPG data. *Comput Electron Agric* 67: 35-42.
- 496 21. Liu X, Williams CE, Nemacheck JA, Wang H, Subramanyam S, et al. (2010) Reactive
497 oxygen species are involved in plant defense against a gall midge. *Plant Physiol*
498 152: 985-999.
- 499 22. Cohen Y, Gisi U (1994) Systemic translocation of ¹⁴C-DL-3-aminobutyric acid in
500 tomato plants in relation to induced resistance against *Phytophthora infestans*.
501 *Physiol Mol Plant Pathol* 45: 441-456.
- 502 23. Jakab G, Cottier V, Toquin V, Rigoli G, Zimmerli L, et al. (2001) β -Aminobutyric
503 acid-induced resistance in plants. *Eur J Plant Pathol* 107: 29-37.
- 504 24. Ton J, Mauch-Mani B (2004) β -Amino-butyric acid-induced resistance against
505 necrotrophic pathogens is based on ABA-dependent priming for callose. *The Plant*
506 *Journal* 38: 119-130.
- 507 25. Will T, van Bel AJE (2006) Physical and chemical interactions between aphids and
508 plants. *J Exp Bot* 57: 729-737.
- 509 26. Tjallingii WF (2006) Salivary secretions by aphids interacting with proteins of phloem
510 wound responses. *J Exp Bot* 57: 739-745.
- 511 27. Howe GA, Jander G (2008) Plant immunity to insect herbivores. *Annu Rev Plant Biol*
512 59: 41-66.
- 513 28. Zhang H, Memelink J (2009) Regulation of secondary metabolism by jasmonate
514 hormones. In: Osbourn AE, Lanzotti V, editors. *Plant-derived Natural Products*:
515 Springer US. pp. 181-194.
- 516 29. Šašek V, Nováková M, Dobrev PI, Valentová O, Burketová L (2012) β -Aminobutyric
517 acid protects *Brassica napus* plants from infection by *Leptosphaeria maculans*.
518 Resistance induction or a direct antifungal effect? *Eur J Plant Pathol* 133:
519 279-289.
- 520 30. Huebner CA, Holthoff K (2013) Anion transport and GABA signaling. *Frontiers in*
521 *Cellular Neuroscience* 7.
- 522 31. Hosie A, Sattelle D, Aronstein K, ffrench-Constant R (1997) Molecular biology of insect
523 neuronal GABA receptors. *Trends Neurosci* 20: 578-583.
- 524 32. Ramputh A-I, Bown AW (1996) Rapid γ -aminobutyric acid synthesis and the inhibition
525 of the growth and development of oblique-banded leaf-roller larvae. *Plant Physiol*
526 111: 1349-1352.
- 527 33. Singh PK, Wu C-C, Zimmerli L (2010) Beta-aminobutyric acid priming by stress
528 imprinting. *Plant Signaling & Behavior* 5: 878-880.

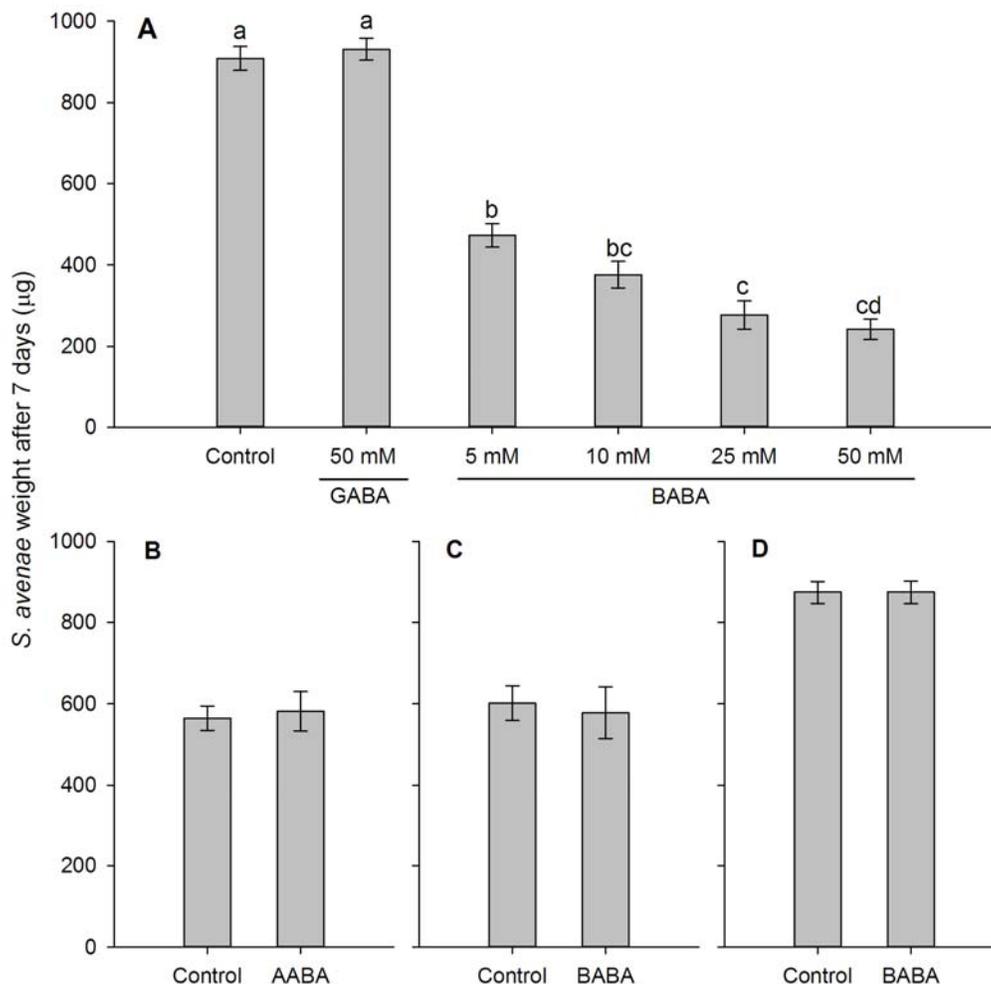
- 529 34. Wu CC, Singh P, Chen MC, Zimmerli L (2010) L-Glutamine inhibits beta-aminobutyric
530 acid-induced stress resistance and priming in *Arabidopsis*. J Exp Bot 61:
531 995-1002.
- 532 35. Schmieden V, Betz H (1995) Pharmacology of the inhibitory glycine receptor: agonist
533 and antagonist actions of amino acids and piperidine carboxylic acid compounds.
534 Mol Pharmacol 48: 919-927.
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573 **Figure Legends**

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576 **Figure 1. *Sitobion avenae* weights after feeding for 7 days on wheat**
577 **seedlings with different treatments.** Aphid weights on wheat plants (A) soil
578 drenched with MilliQ water (control), different concentrations of BABA, 50 mM
579 GABA, (B) soil drenched with MilliQ water and 50 mM AABA, (C) sprayed with
580 MilliQ water and 50 mM BABA, (D) seed treatment with water and 50 mM
581 BABA. Bars represent mean \pm SEM. Different letters above bars in fig. 1A
582 indicate statistically significant differences ($P < 0.05$, Turkey's HSD test).

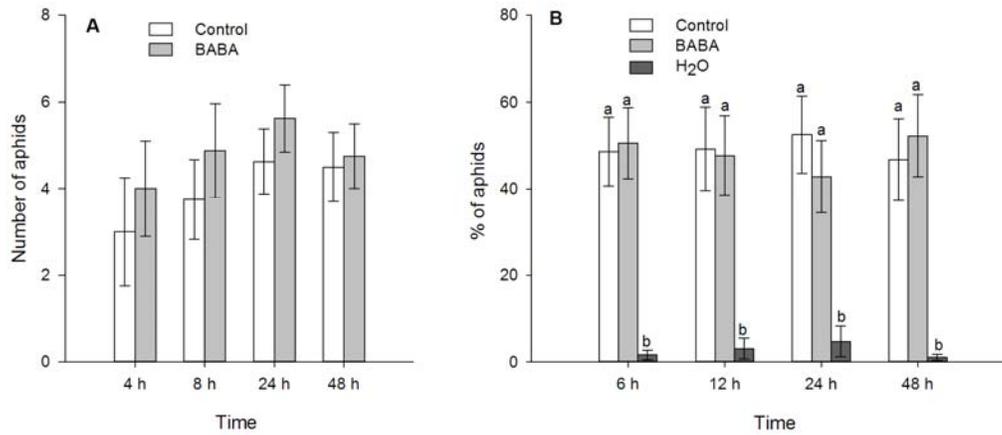


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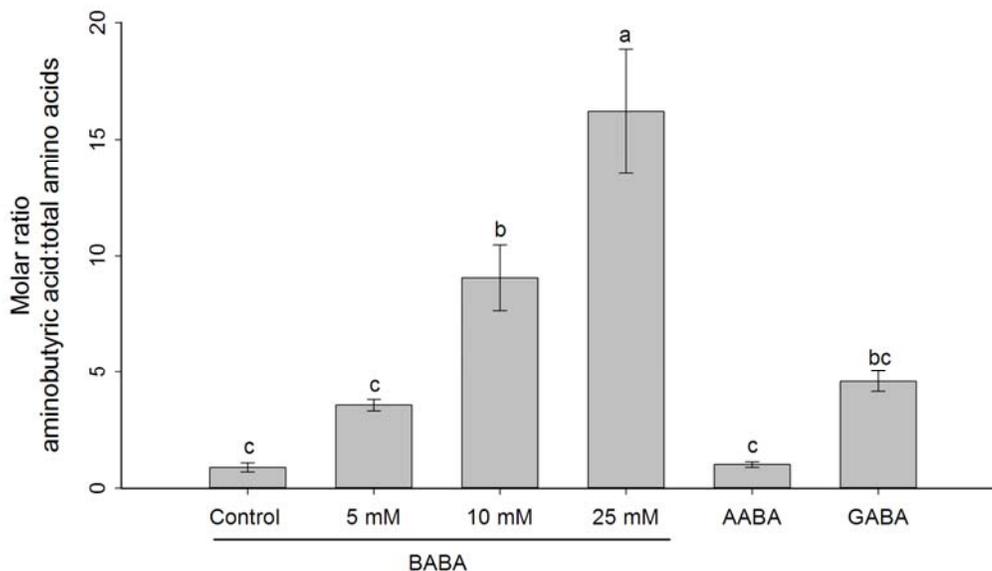
585 **Figure 2. Settling preference of *Sitobion avenae*.** (A) Mean number (\pm SEM)
586 of *S. avenae* settling on control or BABA-treated plants ($n = 8$, paired t -test).
587 Plants were soil drenched with MilliQ water (control) or 25 mM BABA. (B)
588 Percentage of *S. avenae* on MilliQ water (H_2O), standard artificial diet (control),

589 and artificial diet containing 50 mM BABA (BABA). Shown are mean \pm SEM (n
590 = 14). Different letters indicate statistically significant differences ($P < 0.05$,
591 Turkey's HSD test).



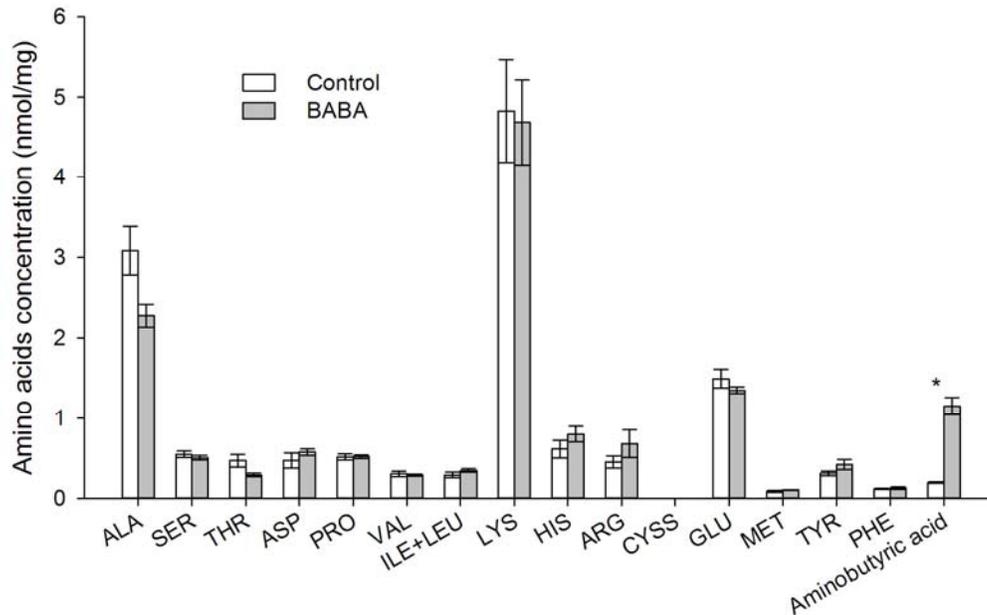
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593 **Figure 3. Relative aminobutyric acid levels in phloem sap of wheat**
594 **seedlings.** Plants were soil drenched with different concentrations of BABA,
595 50 mM AABA and 50 mM GABA. Phloem sap was collected 3 days after
596 treatment. Shown are mean \pm SEM (n = 6). Different letters indicate
597 statistically significant differences ($P < 0.05$, Turkey's HSD test).



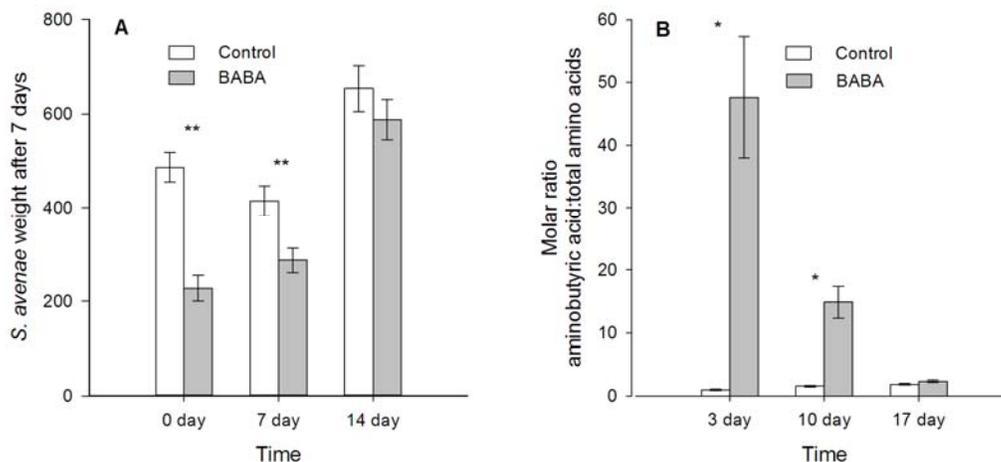
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599 **Figure 4. Amino acids concentration in *Sitobion avenae* bodies.** Wheat
600 plants were soil drenched with MilliQ water (control) and 25 mM BABA. Newly
601 born nymphs feeding on control or BABA-treated plants for 7 days were
602 collected for analysis. Shown are mean \pm SEM (n = 4, * $P < 0.05$, Student's
603 t-test).



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605 **Figure 5. Durability of BABA treatment on *Sitibion avenae* performance**
 606 **and relative aminobutyric content in plant phloem.** (A) Weights of *S.*
 607 *avenae* after feeding on control and BABA-treated plants for 7 days.
 608 Performance assay started 0, 7, and 14 day after treatment. (B) Relative
 609 aminobutyric acid levels in wheat seedlings phloem, which was collected 3, 10,
 610 and 17 days after treatment. Shown are mean \pm SEM (* $P < 0.05$, ** $P < 0.01$,
 611 Student's *t*-test).

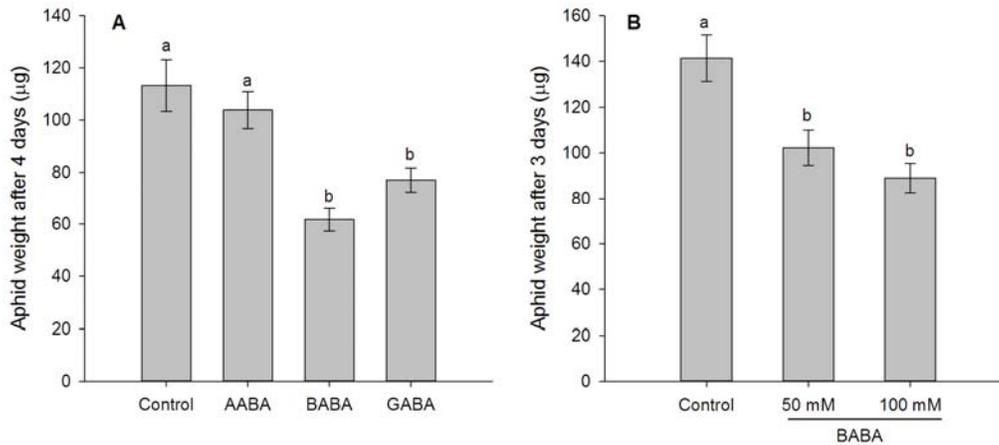


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614 **Figure 6. Direct toxicity of BABA to *Sitobion avenae*.** (A) Weights of
 615 nymphs after feeding on standard artificial diet (control) and artificial diet
 616 containing 50 mM AABA, BABA or GABA for 4 days. (B) Weights of nymphs
 617 after feeding on standard artificial diet (control) and artificial diet containing 50

618 mM BABA or 100 mM BABA for 3 days. Shown are mean \pm SEM. Different
 619 letters indicate statistically significant differences ($P < 0.05$, Turkey's HSD
 620 test).



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625 **Table 1** EPG parameters of *Sitobion avenae* during an 8 h recording on wheat
 626 seedlings soil drenched with water (Control) or beta-aminobutyric acid (BABA)
 627 48 h earlier.

628

EPG Parameters	Control n=21	BABA n=25
Number of probes	10.7 \pm 1.1	13.9 \pm 2.1
Number of short probes (<3 min)	4.0 \pm 0.6	5.5 \pm 1.2
Number of probes to the 1st E1	7.8 \pm 1.3	8.6 \pm 1.0
Time from 1st probe to 1st E1	2.3 \pm 0.4	2.6 \pm 0.3
Total duration of probing (h)	7.1 \pm 0.1	7.0 \pm 0.2
Total duration of pathway	2.1 \pm 0.2	2.3 \pm 0.2
Total duration of E2 (h)	4.1 \pm 0.3	3.8 \pm 0.4
Mean duration of E2 (h)	1.7 \pm 0.3	2.4 \pm 0.5
Total duration of E1 (min)	16.0 \pm 4.1	12.8 \pm 2.3
Mean duration of E1 (min)	5.9 \pm 1.6	5.2 \pm 1.2
Number of E2	3.0 \pm 0.3	2.6 \pm 0.4
Number of E1	3.1 \pm 0.4	2.9 \pm 0.4
Duration of xylem ingestion (h)	0.6 \pm 0.2	0.6 \pm 0.1

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630 Values represent means (\pm SEM). E1: Salivation phase; E2: Phloem sap
 631 ingestion. n: number of replicates.

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