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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Abstract:

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

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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, *Acyrthosiphon 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**
145
146   Plants and Insects
147   Seeds of winter wheat, *Triticum aestivum* (var. XiNong 979), were germinated at room
148   temperature (25 ± 1ºC) 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 ± 1ºC, 60 ± 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

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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 (H_2O), 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 3nd-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.

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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

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

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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).
339
340
341
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

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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 ^{14}C-labled 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 sits 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

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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 *Acyrthosiphon 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 unknowing 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. avnae* 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**

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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 ± SEM. Different letters above bars in fig. 1A
582 indicate statistically significant differences ($P < 0.05$, Turkey's HSD test).

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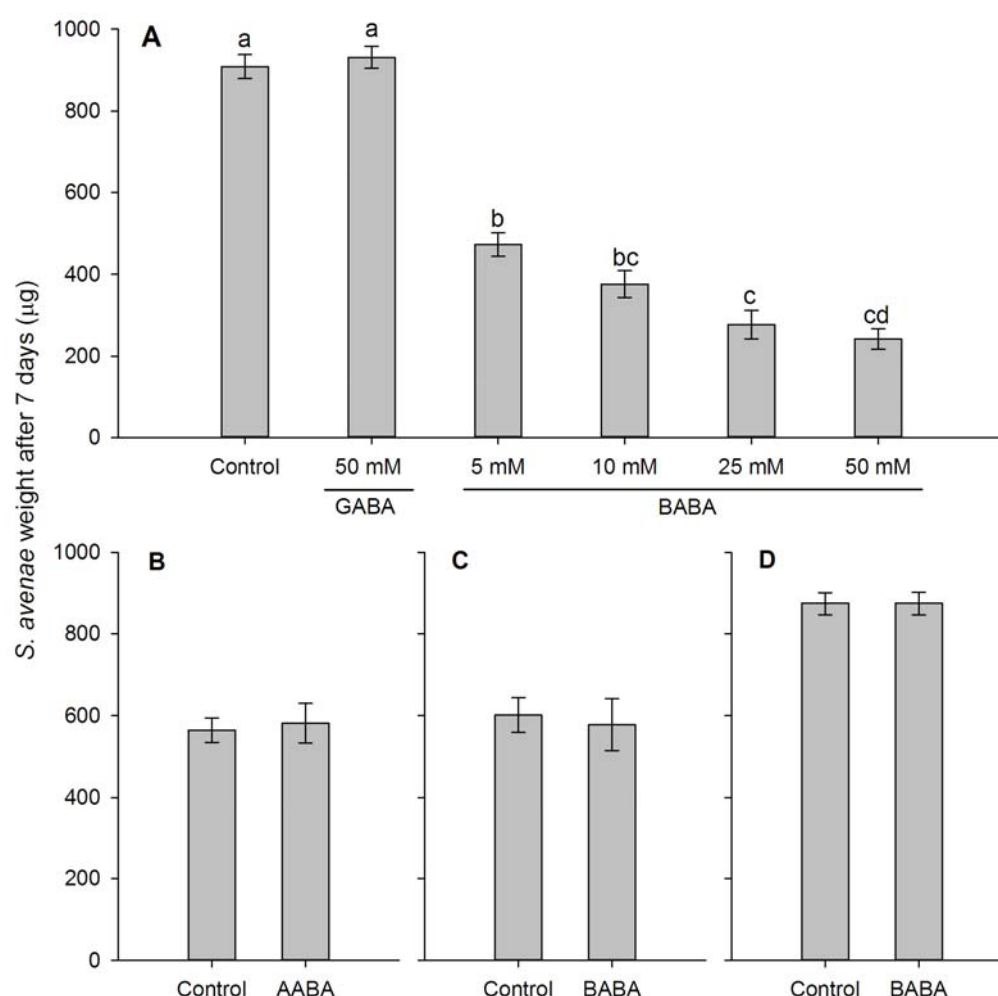
585 **Figure 2. Settling preference of *Sitobion avenae*.** (A) Mean number (± SEM)
586 of *S. avenae* settling on control or BABA-treated plants (n = 8, paired *t*-test).
587 Plants were soil drenched with MilliQ water (control) or 25 mM BABA. (B)
588 Percentage of *S. avenae* on MilliQ water (H_2O),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

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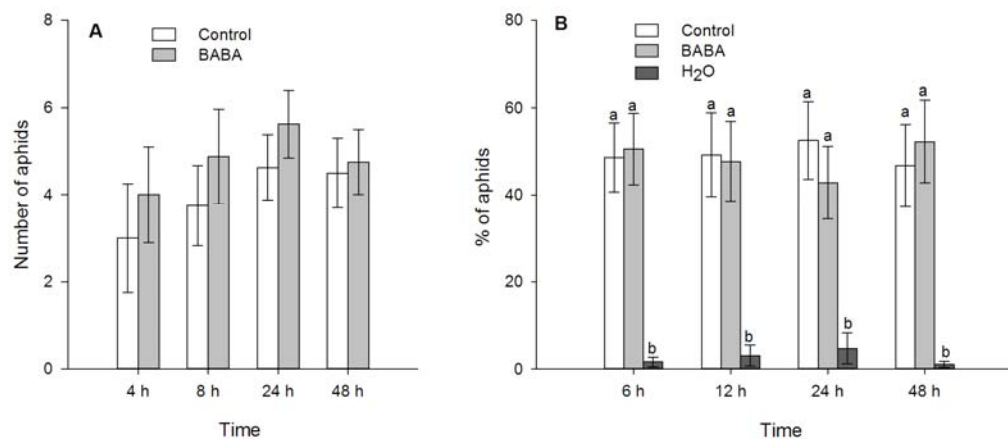
593   **Figure 3. Relative aminobutyric acid levels in phloem sap of wheat**
594   **seedlings.** Plants were soil drenched with different concentrations of BABA,
595   50 mM AABA and 50 mM GABA. Phloem sap was collected 3 days after
596   treatment. Shown are mean ± SEM (n = 6). Different letters indicate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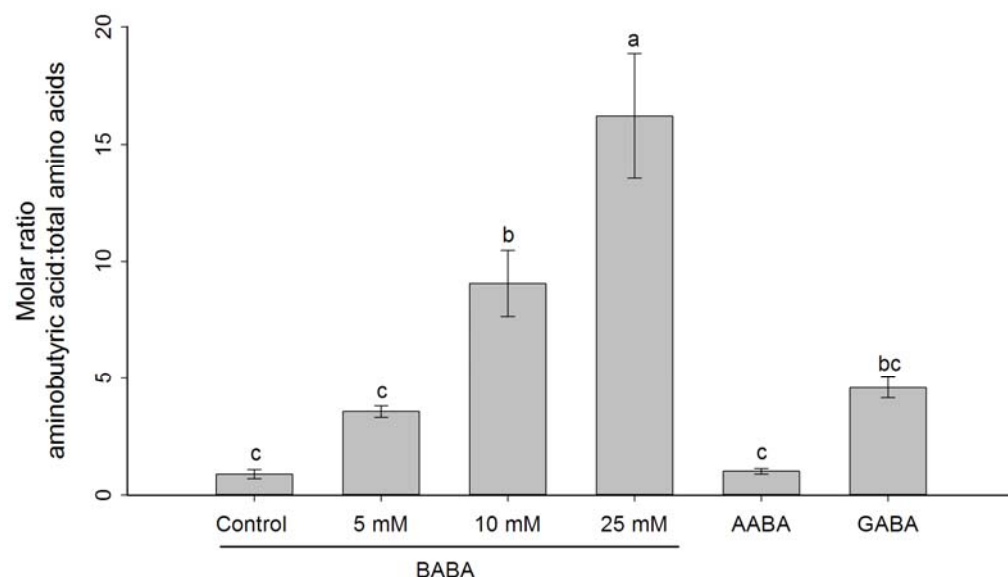

599   **Figure 4. Amino acids concentration in *Sitobion avenae* bodies.** Wheat
600   plants were soil drenched with MilliQ water (control) and 25 mM BABA. Newly
601   born nymphs feeding on control or BABA-treated plants for 7 days were
602   collected for analysis. Shown are mean ± SEM (n = 4, *$P < 0.05$, Student's
603   *t*-test).
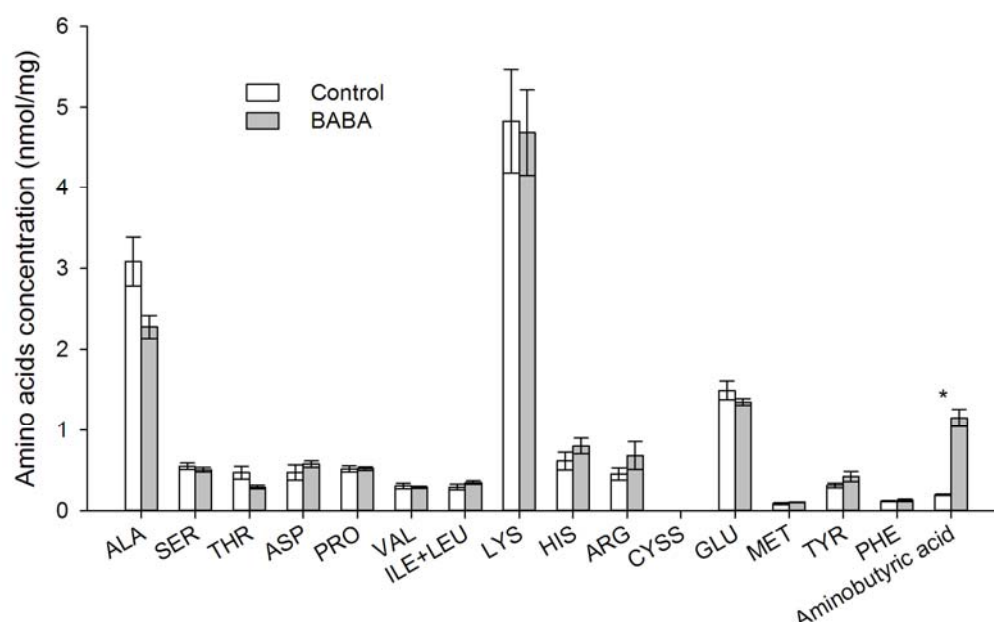
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Figure 5. Durability of BABA treatment on *Sitibion avenae* performance and relative aminobutyric content in plant phloem. (A) Weights of *S. avenae* after feeding on control and BABA-treated plants for 7 days. Performance assay started 0, 7, and 14 day after treatment. (B) Relative aminobutyric acid levels in wheat seedlings phloem, which was collected 3, 10, and 17 days after treatment. Shown are mean ± SEM (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).
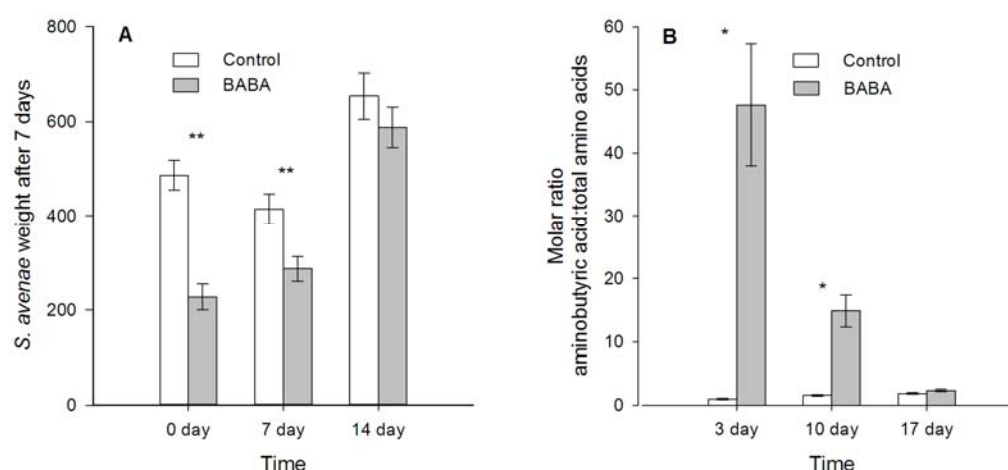

Figure 6. Direct toxicity of BABA to *Sitobion avenae*. (A) Weights of nymphs after feeding on standard artificial diet (control) and artificial diet containing 50 mM AABA, BABA or GABA for 4 days. (B) Weights of nymphs after feeding on standard artificial diet (control) and artificial diet containing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

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618 mM BABA or 100 mM BABA for 3 days. Shown are mean ± SEM. Different
619 letters indicate statistically significant differences ($P < 0.05$, Turkey's HSD
620 test).
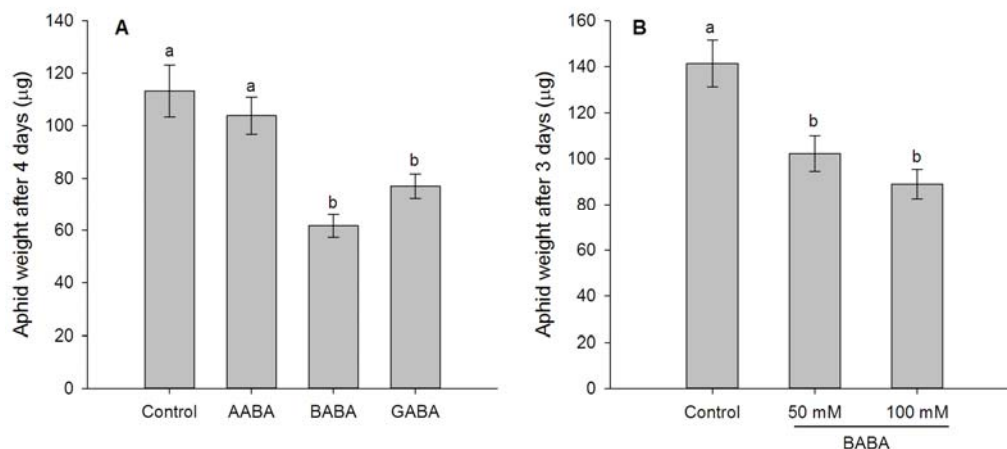
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625 **Table 1** EPG parameters of *Sitobion avenae* during an 8 h recording on wheat
626 seedlings soil drenched with water (Control) or beta-aminobutyric acid (BABA)
627 48 h earlier.
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EPG Parameters	Control n=21	BABA n=25
Number of probes	10.7 ± 1.1	13.9 ± 2.1
Number of short probes (<3 min)	4.0 ± 0.6	5.5 ± 1.2
Number of probes to the 1st E1	7.8 ± 1.3	8.6 ± 1.0
Time from 1st probe to 1st E1	2.3 ± 0.4	2.6 ± 0.3
Total duration of probing (h)	7.1 ± 0.1	7.0 ± 0.2
Total duration of pathway	2.1 ± 0.2	2.3 ± 0.2
Total duration of E2 (h)	4.1 ± 0.3	3.8 ± 0.4
Mean duration of E2 (h)	1.7 ± 0.3	2.4 ± 0.5
Total duration of E1 (min)	16.0 ± 4.1	12.8 ± 2.3
Mean duration of E1 (min)	5.9 ± 1.6	5.2 ± 1.2
Number of E2	3.0 ± 0.3	2.6 ± 0.4
Number of E1	3.1 ± 0.4	2.9 ± 0.4
Duration of xylem ingestion (h)	0.6 ± 0.2	0.6 ± 0.1

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