Induced resistance against the Asian citrus psyllid, *Diaphorina citri*, by \(\beta\)-aminobutyric acid in citrus

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Abstract

\(\beta\)-Aminobutyric acid (BABA) is known to induce resistance to microbial pathogens, nematodes and insects in several host plant/pest systems. The present study was undertaken to determine whether a similar effect of BABA occurred against the Asian citrus psyllid, *Diaphorina citri* Kuwayama, in citrus. A 25mM drench application of BABA significantly reduced the number of eggs/plant as compared with a water control, whereas 200 and 100mM applications of BABA reduced the numbers of nymphs/plant and adults/plants, respectively. A 5mM foliar application of BABA significantly reduced the number of adults but not eggs or nymphs when compared with a water control treatment. In addition, leaf-dip bioassays using various concentrations (25–500mM) of BABA indicated no direct toxic effect on 2nd and 5th instar nymphs or adult *D. citri*. BABA-treated plants were characterized by significantly lower levels of iron, magnesium, phosphorus, sodium, sulfur and zinc as compared with control plants. The expression level of the PR-2 gene (\(\beta\)-1,3-glucanase) in BABA-treated plants that were also damaged by *D. citri* adult feeding was significantly higher than in plants exposed to BABA, *D. citri* feeding alone or control plants. Our results indicate the potential for using BABA as a systemic acquired resistance management tool for *D. citri*.

Keywords: BABA, citrus greening, gene expression, imidacloprid, induced resistance, pathogenesis-related proteins, PR-2 gene, systemic acquired resistance

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Introduction

Several biotic factors, such as beneficial microorganisms, infection by microbial pathogens or infestation by insect pests (Kessler & Baldwin, 2002; Dicke & Hilker, 2003; Pozo et al., 2004), can induce resistance in plants against diseases and insect pests (van der Ent et al., 2009; Cohen et al., 2010). Induced resistance can also be activated by chemicals such as \(\beta\)-aminobutyric acid (BABA), a non-protein amino acid (Zimmerli et al., 2000). BABA has been documented to induce plant resistance mechanisms in the following host/pest systems: *Bremia lactucae*, an Oomycete, on lettuce (Cohen et al., 2010, 2011); *Phytophthora infestans* on tomatoes (Cohen et al., 1994); *Acrthosiphon pisum* (Harris), a pea aphid, on legumes (Hodge et al., 2005); *Myzus persicae* (Sulz.), *Brevicoryne brassicae* (L.), *Trichoplusia ni* (Hübner) and *Plutella xylostella* (L.) on several species in the Brassicaceae (Hodge et al., 2006); and *Heterodera avenae* Woll., *Heterodera latipons* and *Meloidogyne* sp., which are plant parasitic nematodes, on wheat and barley (Oka & Cohen, 2001). In addition, BABA is known to induce resistance against abiotic stresses such as drought, heat, salinity and acid rain (Jakab et al., 2001; Liu et al., 2011).

BABA induces resistance in plants through a number of physical and biochemical mechanisms (Cohen, 2002). Accumulation of pathogenesis-related (PR) proteins is one of the responses elicited by the application of BABA to plants (Cohen et al., 1994; Hwang et al., 1997). Expression of systemic acquired resistance (SAR) in plants as a result of BABA...
application is related to elevated expression levels of the PR-1, PR-2, and PR-5 genes (Jakab et al., 2001). BABA-related elevation of PR protein levels can occur in tomato, pepper and tobacco plants in the absence of biotic stress by a plant pathogen (Cohen et al., 1994; Hwang et al., 1997), whereas in crucifers, PR protein levels were elevated only after the addition of biotic stress of pathogen infection (Zimmerli et al., 2000, 2001; Silue et al., 2002). BABA also increases lignin and callose content in foliar tissues and vascular walls, respectively, which can adversely affect feeding by herbivores (Cohen et al., 1999; Hamiduzzaman et al., 2005). Another physical change imposed by BABA is the alteration of nutrient composition and dry-matter content in the host plant (Hodge et al., 2005). However, little is known about the role of BABA on the level of PR proteins and plant nutrients that are known to influence the development of induced resistance against insect pests.

The objective of this study was to determine whether a soil drench application of BABA (Sigma-Aldrich, Inc., St Louis, MO, USA; purity 97%) affected development of *D. citri* on citrus. The first experiment was conducted between 6 May 2010 and 3 June 2010. The treatments tested were 25, 50 or 100 mM of BABA dissolved in distilled water compared with a water only control. A second complementary experiment was conducted between 19 August 2010 and 17 September 2010. The treatments compared were 25, 100, 200 or 500 mM of BABA compared with a water control. Each experiment was arranged in a randomized complete block design and there were seven and ten replicates during the first and second experiments, respectively. Each BABA concentration was prepared in distilled water, and 25 ml of each concentration was applied as a drench to each plant. Plants were allowed to prime for 3 days after drenching under controlled greenhouse conditions. Thereafter, five male and five female *D. citri* adults of similar age were released onto each plant for 5 days. Each plant was covered with a perforated plastic cup to contain the *D. citri* adults. After 5 days, all of the adults were removed from each plant, and the number of eggs per plant was counted. Subsequently, the numbers of nymphs and adults produced per plant were counted semiweekly. Plants were observed for signs of phytotoxicity throughout the experiment.

The termination of the second experiment, eight plants that received either 500 mM of BABA or water control were randomly selected for macro- and micronutrient analysis. Plant tissues were washed, air-dried and ground to pass through a 0.38-mm sieve. A plant tissue sample comprising approximately 3 g plant material was shipped to Waters Agricultural Laboratories, Inc. (Camilla, GA, USA) for nutrient analysis. Phosphorus, potassium, calcium, magnesium, sulfur, manganese, iron, copper, zinc, boron, silicon, sodium, molybdenum, nitrate, aluminum and chlorine concentrations were determined by inductively coupled plasma atomic emission spectroscopy.

For each experiment, the total number of eggs, nymphs and adults per plant were analyzed separately by analysis of variance (ANOVA) (PROC GLM) using BABA concentration as the main effect, followed by Fisher’s protected LSD mean separation (SAS, 2004). The level of each nutrient was compared between BABA-treated and control plants using
ANNOVA, followed by Fisher’s protected LSD mean separation (SAS, 2004).

Effect of BABA foliar application on developmental stages of D. citri

The objective of this experiment was to examine the effects of foliar applications of BABA on developmental stages of D. citri. The experiment was conducted twice between 8 March 2011 and 22 April 2011, and between 6 May 2011 and 3 June 2011. Each experiment consisted of the following treatments: 5mM BABA; 625μl 1⁻¹ of imidacloprid (Provado 1.6 F, Bayer CropScience, Research Triangle Park, NC, USA) (positive control); or water (negative control). Each experiment was arranged as a randomized complete block design with ten replicates of each treatment. Imidacloprid was used at the full (625μl 1⁻¹) recommended label rate against D. citri. BABA and imidacloprid were prepared in distilled water. Twenty-five milliliters of each treatment were applied as a foliar application until runoff using a handheld atomizer (The Bottle Crew, West Bloomfield, MI, USA). Plants were allowed to prime for 3 days after treatment application under controlled greenhouse conditions. Thereafter, five male and five female D. citri adults of similar age were released onto each plant and held for 5 days. Each plant was covered with a perforated plastic cup to contain the adults. Thereafter, all of the adults were removed from each plant, and the number of eggs per plant was counted. Subsequently, the numbers of nymphs and adults produced per plant were counted semiweekly. Plants were observed for any signs of phytotoxicity throughout the experiment. Owing to the lack of a significant effect of time on the main response variables, data from both experiments were pooled to analyze the total number of eggs, nymphs and adults per plant using ANOVA, followed by Fisher’s protected LSD mean separation (PROC GLM) (SAS, 2004).

D. citri toxicity bioassay

The direct toxicity of four concentrations of BABA (25, 100, 200 and 500 mM) on early (second) and late (fifth) instar nymphs, and adults was determined using a Petri-dish bioassay method (Prabhaker et al., 2006; Tiwari et al., 2011b) and compared with water alone (negative control) and one-tenth of the recommended label rate of imidacloprid (positive control). The bioassay arena consisted of a 60-mm-diameter plastic disposable Petri-dishes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA, USA) containing a 2–3 mm thick solidified bed of 1.5% agar solution. Leaf disks (60 mm diameter) from fresh citrus leaves were excised, dipped for 30 s in BABA solutions made in water and allowed to air dry in a fume hood for 1 h prior to bioassays. For the negative control treatment, leaf disks were dipped in distilled water alone. For the positive control treatment, leaf disks were dipped in an imidacloprid formulation dissolved in distilled water. After 1 h, leaf disks were placed on agar beds and 20–30 nymphs or adults were transferred into each dish using a camel hair brush. The adults were briefly anesthetized with CO2 to facilitate handling and transfer. Petri-dishes were wrapped with parafilm (Pechiney Plastic Packagin, Chicago, IL, USA) to prevent the escape of psyllids. Sealed Petri-dishes with nymphs or adults were transferred into a growth chamber (Percival Scientific, Inc., Perry, IA, USA) set at 25±1°C, 50±5% RH and 14:10 (L:D) photoperiod. Each BABA concentration was replicated three times (n=30–45 D. citri per concentration) and the entire experiment was repeated. The mortality of nymphs or adults was assessed 48 h after placement into the growth chamber. Adults found on their side or back that were unable to move when probed with a camel hair brush were considered dead. Nymphs found flaccid, dried, light colored and unable to move when probed with a camel hair brush were considered dead. Percent mortality between treatments was compared separately for each developmental stage using ANOVA (PROC GLM), followed by Fisher’s protected LSD mean separation (SAS, 2004).

PR-2 gene (β-1,3-glucanase) expression levels in BABA-treated plants

The expression level of the PR-2 gene was evaluated in citrus after receiving one of the following four treatments: BABA application (500mM); BABA application (500mM) on plants exposed to D. citri feeding; plants exposed to D. citri feeding alone; and a control (no exposure of plants to D. citri or BABA). Each treatment was replicated three times. This experiment was conducted using potted ‘Swingle’ plants under controlled conditions on 18 October 2011. Each potted plant was covered with a perforated plastic cup to contain adults on the plants and to prevent external infestation. In the treatment where plants were exposed to D. citri adults, five pairs of similarly aged D. citri adults were released onto each plant. After 5 days, adults were removed from the plants, and 25 ml of 500mM BABA solution was applied as a drench to each plant receiving treatments of BABA. Control plants or those receiving D. citri alone were treated with 25 ml of water alone. Plants were allowed to prime for 3 days. Thereafter, leaves from each plant were used to evaluate PR-2 gene expression levels. Total RNA was extracted from 200 mg of tissue from each treatment using the RNeasy Mini Kit for plant tissue (Qiagen, MD, USA). The quantity and quality of RNA from each sample was measured on a NanoDrop 1000 Spectrophotometer using the A₂₆₀ and the A₂₆₀/A₂₈₀ ratio to ensure uniform quality and quantity (100 ng μl⁻¹) among all treatments for subsequent real-time reverse transcription PCR (RT–PCR) analysis. One microliter of RNA sample was used for RT–PCR using β-1,3-glucanase primers (Forward=TTCCACTGCATGCACACCTG; Reverse=TTGTTATCTTGTTTTAAATGAGCCTCTTG) (Francis et al., 2009). RT–PCR reactions were conducted using a Power SYBR Green Reverse Transcription kit (Applied Biosystems, Foster City, USA) and a temperature cycle consisting of 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Three biological replicates were used per treatment.

To compare relative expression of the PR-2 gene among treatments, we used the 2⁻ΔΔCt method (Livak & Schmittgen, 2001) by normalizing to expression of the plant cytochrome oxidase (COX) gene (Forward=GTTATGCCCAGTGCCCATC- TCCAGA; Reverse=GCCAAAACGTCTAAGGCCATT) (Li et al., 2006), followed by normalization to the treatment giving the lowest gene expression. ANOVA was performed to compare the relative expression of the PR-2 gene among plants receiving various treatments (PROC GLM) (SAS, 2004).

Results

Effect of BABA drench applications on developmental stages of D. citri and plant nutrients

In the first experiment, BABA significantly affected the mean numbers of eggs laid (F=6.76; df=3, 36; P=0.0010),
nymphs \( (F=3.73; \text{df}=3, 36; \ P=0.0196) \) and adults produced \( (F=5.39; \text{df}=3, 36; \ P=0.0036) \) per plant (fig. 1a–c). The mean numbers of eggs (fig. 1a), nymphs (fig. 1b) and adults (fig. 1c) on plants receiving 25 mM BABA were significantly reduced by over 50%, 35% and 42%, respectively, compared with control plants. In the second experiment, BABA significantly affected the mean numbers of eggs laid \( (F=3.51; \text{df}=4, 30; \ P=0.0182) \), nymphs \( (F=3.39; \text{df}=4, 30; \ P=0.0212) \) and adults produced \( (F=2.87; \text{df}=4, 30; \ P=0.0400) \) per plant (fig. 2a–c). The mean number of eggs laid per plant was significantly reduced by over 72% on plants receiving 25 mM BABA compared with control plants (fig. 2a). The mean numbers of nymphs and adults were reduced by over 60% and 84% on plants receiving 100 and 200 mM BABA, respectively, when compared with control plants (fig. 2b, c). During both experiments, the control plants had significantly higher numbers of eggs, nymphs and adults per plant when compared with the highest concentrations of BABA tested (figs 1a–c and 2a–c).

Nutrient analysis revealed that levels of iron \( (F=9.01; \text{df}=1, 14; \ P=0.0095) \), magnesium \( (F=5.03; \text{df}=1, 14; \ P=0.0416) \), phosphorus \( (F=29.07; \text{df}=1, 14; \ P<0.0001) \), sodium \( (F=13.10; \text{df}=1, 14; \ P=0.0028) \), sulfur \( (F=7.78; \text{df}=1, 14; \ P=0.0145) \) and zinc \( (F=12.71; \text{df}=1, 14; \ P=0.0031) \) were significantly higher in untreated control plants compared with BABA-treated plants (table 1). No significant difference was found in levels of aluminum, boron, calcium, chloride, copper, manganese, molybdenum, potassium and silica.

Fig. 1. Mean number of *D. citri* eggs (A), nymphs (B) and adults (C) produced per citrus plant, following drench application of BABA during spring 2010.
between untreated control and BABA-treated plants ($P > 0.05$) (table 1).

**Effect of BABA foliar applications on developmental stages of D. citri**

Treatments involving foliar applications of BABA, imidacloprid (full recommended rate) and water alone were found to significantly affect the mean numbers of eggs ($F = 11.09$; df = 2, 57; $P < 0.0001$), nymphs ($F = 12.66$; df = 2, 57; $P < 0.0001$) and adults ($F = 15.19$; df = 2, 57; $P < 0.0001$) per plant (fig. 3). A 5 mM BABA alone treatment significantly reduced the number of adults, but not eggs or nymphs when compared with the control treatment. BABA treatments reduced egg, nymph and adult production by 24%, 33% and 60%, respectively, when compared with the control. The imidacloprid positive control reduced egg, nymph and adult production by 96%, 96% and 83%, respectively, when compared with water alone (negative control).

**D. citri toxicity bioassay**

BABA treatments did not cause mortality of *D. citri* as compared with the control (table 2). Mortality of 2nd instar ($F = 362.17$; df = 5, 37; $P < 0.0001$), 5th instar ($F = 543.85$; df = 5, 37; $P < 0.0001$) and adult ($F = 827.24$; df = 5, 37; $P < 0.0001$). *D. citri* was significantly greater in treatments receiving imidacloprid (positive control) as compared with the BABA or water (negative control) treatments (table 2).

**PR-2 gene ($β$-1,3-glucanase) expression levels in BABA-treated plants**

The objective of this experiment was to determine whether exposure of citrus plants to *D. citri* adult feeding alone or in combination with BABA treatment induces expression of the PR-2 gene (fig. 4). Significant variation in the expression level of the PR-2 gene was observed among treatments ($F = 6.84$; df = 3, 20; $P = 0.0024$). Significantly higher expression of PR-2 was observed in plants exposed to 500 mM of BABA in combination with *D. citri* adult feeding when compared with the control or other treatments tested.

**Discussion**

The current results demonstrate that treating citrus plants with BABA can negatively impact all three developmental stages of *D. citri*. During the first experiment, drench applications with as little as 25 mM of BABA significantly reduced the number of eggs, nymphs and adults per plant. However, during the second drench application experiment, 25 mM of BABA significantly reduced the number of eggs, while 100 and 200 mM of BABA reduced the number of adults and nymphs surviving, respectively, compared with the control treatment. Foliar application of BABA was more effective than drench application, with respect to reducing the...
number of adults with 5mM of BABA. Our results indicate that the application method affects the impact of BABA on development of *D. citri*.

The mechanism(s) of BABA-induced resistance in plants against insects are not well understood in general. Therefore, we sought to determine whether BABA was directly toxic to *D. citri*. Our results proved no direct toxicity of BABA against any of the developmental stages of *D. citri* tested suggesting an induced resistance response by citrus. The lack of acute toxicity found in this study has also been reported for BABA in other insect species such as aphids, *A. pisum* and *M. persicae* (Hodge et al., 2005), and plant pathogenic nematodes, *H. avenae, H. lapitons* and *Meloidogyne* sp. (Oka & Cohen, 2001). The deleterious effects of BABA on development of *D. citri* might be related to decreased nutritional quality of host plants given significantly lower levels of iron, magnesium,

![Fig. 3. Mean number of *D. citri* eggs (A), nymphs (B) and adults (C) produced per citrus plant, following foliar application of BABA. BABA was applied at the concentration of 5mM and imidacloprid was applied at the recommended label rate of 625μl⁻¹.](image-url)
Table 2. Mean percent mortality of three developmental stages of Asian citrus psyllid, *D. citri*, following direct contact with BABA or imidacloprid.

<table>
<thead>
<tr>
<th>Developmental life stage</th>
<th>Imidacloprid</th>
<th>Mean (±SEM) percent mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0mM</td>
<td>25mM</td>
</tr>
<tr>
<td>Second instar nymph</td>
<td>87.7±0.7a</td>
<td>3.1±0.9b</td>
</tr>
<tr>
<td>Fifth instar nymph</td>
<td>93.0±3.9a</td>
<td>2.5±1.0b</td>
</tr>
<tr>
<td>Adult</td>
<td>94.1±3.0a</td>
<td>2.9±0.9b</td>
</tr>
</tbody>
</table>

Mean mortality percentage values followed by the same letter within each developmental stage (row) are not significantly different (P<0.05).

Phosphorus, sodium, sulfur and zinc in BABA-treated plants as compared with control plants. BABA-treated *Vicia faba* L. plants exhibit altered dry-matter content and C, N and H content of leaves, which detrimentally impacts production of *L. plants* exhibit altered dry-matter content and C, N and H phosphorus, sodium, sulfur and zinc in BABA-treated plants as a result of the combined effect of BABA and or BABA).

**Fig. 4.** Relative expression levels of the PR-2 gene in plants exposed to BABA alone (500 mM); BABA (500 mM) and *D. citri* feeding; *D. citri* feeding alone; and control (no exposure of plants to *D. citri* or BABA). Ct values obtained from real-time RT-PCR were first normalized to the reference gene, plant COX, followed by normalization to the treatment giving the lowest gene expression using the 2^ΔΔCt method. Values sharing the same letter are not significantly different (P<0.05; Fisher’s protected LSD).

Induced resistance against the Asian citrus psyllid, *D. citri*, by BABA in citrus

**Table 2.** Mean percent mortality of three developmental stages of Asian citrus psyllid, *D. citri*, following direct contact with BABA or imidacloprid.

Mean mortality percentage values followed by the same letter within each developmental stage (row) are not significantly different (P<0.05).

Also possible that this could result in the need for fewer annual insecticide applications. This may be especially important given the recent emergence of insecticide resistance in populations of *D. citri* in the U.S. (Tiwari et al., 2011a).

Even the highest concentration of BABA (500 mM) tested in the present study caused no phytotoxic effects to citrus plants. The lack of phytotoxicity observed with citrus is similar to that seen with pea (*Pisum sativum*), broad bean (*Vicia faba* var. major), runner bean (*Phaseolus coccineus*), red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) (Hodge et al., 2005).

However, BABA does cause necrosis in tobacco (Cohen & Gisi, 1994; Siegrist et al., 2000). The application technique can influence phytotoxicity caused by BABA (Cohen, 1994; Cohen & Gisi, 1994). BABA concentrations as low as 1 and 10 mM were found to cause necrotic lesions on tobacco when applied as foliar sprays (Cohen, 1994; Siegrist et al., 2000). However, tomato plants tolerate higher concentrations of BABA when applied as a soil drench (Cohen & Gisi, 1994), because they systemically acquire only part of the applied volume. Citrus appears to tolerate BABA applications better than tobacco due to a lack of phytotoxicity after both drench and foliar applications.

Mechanisms for BABA-induced resistance in plants vary among plant families and pest/pathogen types (Cohen, 2002; Marcucci et al., 2010). In grapes such as *Vitis vinifera*, BABA-induced resistance is mediated by the phenylpropanoid and jasmonic acid pathways (Hamiduzzaman et al., 2005; Slaughter et al., 2008). BABA is also known to induce resistance through enhanced expression of systemically acquired resistance (SAR) genes, which code for pathogenicity-related (PR) proteins (Cohen et al., 1994; Hwang et al., 1997; Hamiduzzaman et al., 2005). BABA-induced resistance in pepper, *Capsicum annuum*, occurs through the accumulation of PR proteins, such as β-1,3-glucanase, chitinase isoforms and other salicylic acid-dependent PR proteins (Hwang et al., 1997). BABA-induced resistance is also regulated by the plant hormone, salicylic acid and the defense regulatory protein, NPR1 (Zimmerli et al., 2000; Ton et al., 2005). In citrus, SAR induction was explained by high expression of the PR-2 gene, which in turn relates to a higher resistance of citrus against canker (Francis et al., 2009). The results of the present study show that the PR-2 gene was up-regulated by more than 150-fold in citrus treated with BABA in combination with *D. citri* adult feeding compared with the control or citrus treated with BABA or *D. citri* feeding alone. These results suggest that PR proteins, or at least one PR protein in citrus, accumulates as a result of the combined effect of BABA and *D. citri* feeding. Our results corroborate findings of another study, where green peach aphid, *M. persicae*, feeding resulted in the activation of defense-related genes, including PR-1 and
BGL2 in Arabidopsis (Moran & Thompson, 2001). Lack of elevated PR-2 gene expression in BABA treatment could have occurred because the concentration of BABA tested may have not been sufficiently high to cause gene expression. Alternatively, the interval between BABA treatment and our assay may have not been sufficiently long to cause PR-2 gene up-regulation. Therefore, optimizing the concentration and priming period of BABA is needed to further elucidate the mechanisms imparting induced resistance in citrus after treatment with BABA. Also, future investigation of the involvement of the jasmonic acid pathway in SAR in citrus is warranted, particularly as pertaining to induced resistance against D. citri.

Our results show that all three developmental stages of D. citri were negatively impacted by BABA through induction of host–plant resistance in citrus. These results suggest an additional viable alternative tool for current D. citri management programmes that heavily rely on insecticides. The synergistic effect of BABA mixed with imidacloprid or other commonly used insecticides requires investigation for the potential use of BABA as a tank-mix with other insecticides for managing D. citri. BABA may also be useful as a tank-mix with other pesticides, given its known synergistic effects with various plant activators and fungicides (Zhang et al., 2001; Cohen, 2002). Additional investigations are needed to optimize a cost-effective and efficacious dosage of BABA for management of D. citri under field conditions. The effects of BABA should also be investigated on other insect pests or diseases of citrus and/or other crops. The current results also suggest the potential for investigating other commercially available SAR-inducing products, such as 2,6-dichloroisocicotinic acid, benzothiadiazole, inorganic salts and salicylic acid, which may induce resistance in citrus against insect pests and pathogens. All the above-mentioned products are known to induce resistance in plants against various pests and pathogens (Edreva, 2004).

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References


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