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Homobrassinolide Delays Huanglongbing Progression in Newly Planted Citrus Trees

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Abstract: Huanglongbing (HLB), or citrus greening, is a devastating disease impacting citrus trees worldwide, with severe effects particularly noted in Florida. Current strategies to combat HLB have focused on aggressive replanting despite the high susceptibility of young trees to infection. In this context, it is critical to explore agronomic practices that can enhance the health and resistance of young citrus trees to HLB. Here, we demonstrate that treatment with homobrassinolide (HBr), a type of brassinosteroid, on newly planted citrus trees can delay HLB infection and improve tree health amidst the high psyllid pressure conditions endemic to Florida. Our study reveals a significant reduction in HLB infection rates in HBr-treated trees compared to control trees, with only 25% of treated trees testing positive for HLB by six months, in contrast to 100% infection in untreated trees. This delay in infection may be attributed to HBr inducing an immune response and negatively impacting psyllid performance, as subsequently demonstrated in a greenhouse experiment. Our findings suggest that HBr applications could serve as a viable strategy to enhance the resilience of citrus production against HLB, underscoring the need for further investigation into their mechanisms of action and potential role in a comprehensive pest and disease management strategy.

Keywords: Brassinosteroids; citrus greening, *Diaphorina citri*; immune response; infection rates; plant health

1. Introduction

Huanglongbing (HLB), also known as citrus greening, is the main threat facing citrus worldwide [1,2]. In Florida, the disease is associated with the gram-negative α-proteobacterium, *Candidatus* Liberibacter asiaticus (CLas), with the Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) being the natural vector [3–5]. Currently, HLB is considered endemic in Florida [6]. Since the disease was first detected in 2005, citrus acreage and fruit production have been reduced by more than 70% in Florida [6,7] and continue to decline. The bacteria propagate within the phloem of citrus trees, producing blotchy mottle and chlorotic patterns on leaves, callose deposition in phloem, canopy die-back, root loss, poor-quality fruit, and premature fruit drop [8–10]. The decline of mature trees over the years due to the disease has driven the industry to adopt a very aggressive replanting strategy of new trees, either in whole blocks after removing the dead trees or by replacing individual affected trees (resetting). The risk of new plantings becoming infected is exceptionally high because young trees frequently flush, attracting more psyllids. These psyllids feed on the new flushes and transmit the bacteria to the plant's vascular system, making them more vulnerable to infection than mature trees [11,12]. This is particularly important in the case of reset trees, as the surrounding environment usually contains mature declining trees that are heavily infected, which act as a source of inoculum for the psyllids. In this scenario of widespread infection and severe

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symptoms of HLB in Florida, agronomic practices that improve plant health in the presence of the disease are needed. It has been shown that epibrassinolide, a form of brassinosteroid (Br), can reduce bacterial titers and alleviate symptoms of greening in HLB-affected citrus trees in trials in Cuba. The effect seems to be mediated by the activation of many defense-related pathways in the tree [13]. Brs are a unique class of polyhydroxylated steroidal phytohormones. Among the more than 70 types of Br reported so far, 24-epibrassinolide and 28-homobrassinolide are the two most active forms of Brs, which are also available commercially [14]. Br signaling was initially studied in the context of growth and development. Still, more recently functional roles of Br in stress responses have been accumulating [15–17], and several crosstalk models of Br signaling and immune signaling have been proposed. Brs apparently integrate immune system function with normal growth and developmental programs, thus serving as essential regulators of the innate trade-off between disease resistance and plant growth. This seems to be plant-species specific, with disparate effects [18]. The role of Brs in plant responses to pathogens also appears to be complex, as the regulation of immunity occurs at multiple levels [19–21]. Recently, it has been demonstrated that Brs can coordinate and enhance salicylic acid-mediated immune responses in *Arabidopsis thaliana* [22]. Interestingly, the interplay between jasmonic acid (JA) and Brs in modulating responses to phloem-feeding insects has also been recently elucidated in plants. For instance, Br induces JA in response to phloem-feeding planthoppers in rice [23], and in citrus Canales et al. [13] have shown that allene oxidase synthase, a key enzyme in the JA synthesis pathway, is induced by epibrassinolide treatment.

In Florida, a formulation of homobrassinolide is available and labeled for use in citrus at the commercial level. Studies on the effects of homobrassinolides (HBr) on responses to plant diseases or biotic stress are still scarce, and to the best of our knowledge, such studies have not yet been conducted in citrus. In this work, we provide the first evidence that HBr, when applied to newly planted citrus trees in an area of high psyllid pressure and endemic HLB conditions, can reduce the rate of HLB infection and improve tree performance. The possible mechanisms of action are presented and discussed.

2. Results

2.1. Field Experiment

In this work, we have assessed the effect of HBr treatments on HLB incidence in newly planted citrus trees. Trees were planted from the nursery when they were 18 months old. Then, trees received HBr treatment monthly. After planting, 80% of trees that did not receive the HBr treatment tested positive for HLB by 6 months, and by 1 year, all trees were infected (**Figure 1**). In contrast, only 25% of trees treated monthly with HBr tested positive for HLB by 6 months, and still some trees (15%) were HLB-negative after 1 year (**Figure 1**). After one year post-transplantation, during which the trees had not received any pesticide treatment, it was decided to assess the status of two key pests in Florida citrus cultivation: the citrus rust mite (CRM), *Phyllocoptruta oleivora* (Ashmead) (Acari: Eriophyidae) and the ACP. To do this, the attack of the CRM was evaluated first by counting the number of mites per surface and the percentage of surface scarred by CRM on the fruit peel. The number of CRM was similar in both treatments (t = 0.5838; df = 1, 8; P = 0.575; **Figure 2a**), but the scarred surface was significantly smaller in fruits sampled from HBr-treated trees compared to those from the control (t = 3.800; df = 1, 8; P = 0.0052; **Figure 2b**). To sample ACP, an initial adult population was estimated using stem-tap sampling, which yielded similar results in both treatments (t = 0.138; df = 1, 8; P = 0.894; **Figure 2c**). However, upon further evaluation of the number of eggs, nymphs, and adults present per tender flush, a lower number of individuals were found in the flushes of HBr-treated- compared to the control (t = 2.358; df = 1, 8; P = 0.046; **Figure 2d**).

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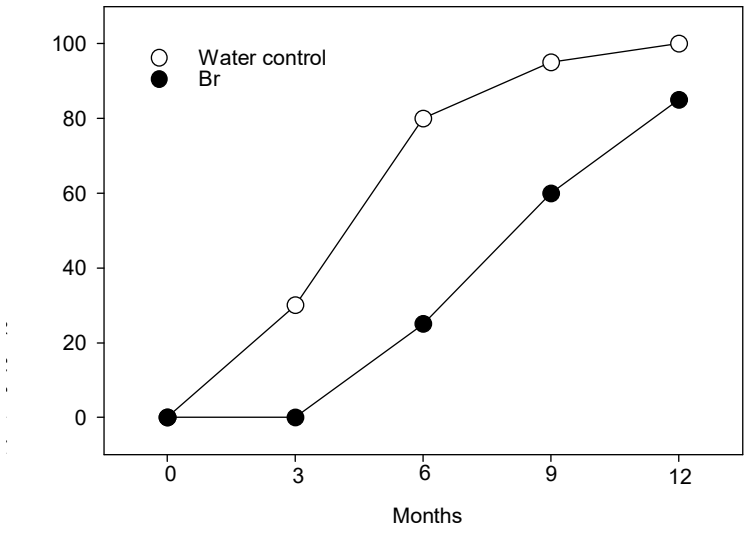

Figure 1. Percentage of trees infected by HLB in HBr-treated plants and control plants in the field trial located in the experimental plots of SWFREC.

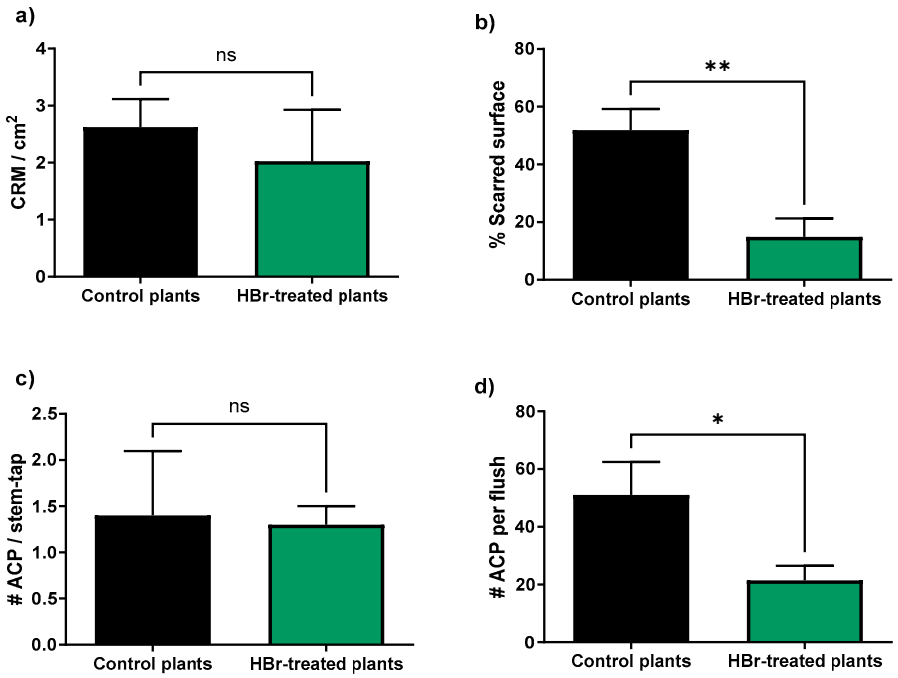

Figure 2. Number (mean ± SE) of citrus rust mites (CRM) per square centimeter of orange fruit peel surface (**a**), Percentage (mean ± SE) of scarred surface area of orange fruit peel (**b**), number (mean ± SE) of adults of D. citri (ACP) per stem-tap sampling (**c**), number (mean ± SE) of eggs, nymphs, and adults of D. citri per flush (**d**), for HBr-treated plants and control plants in the field trial located in the experimental plots of SWFREC. * and ** indicate significant differences at the 5%, 1%, and 0.01% levels by t-test. "ns" indicates no significance by t-test.

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2.2. Greenhouse Experiment

To confirm the results obtained in the field and delve deeper into the effects that HBr treatments may have on the ACP, we decided to set up a greenhouse experiment under controlled conditions. To accomplish this, we released 5 pairs of *D. citri* per two-year-old plant (Valencia grafted onto X-639). We assessed their performance on plants previously treated with HBr, followed by weekly treatments, comparing them with control plants treated with water. The number of *D. citri* individuals that developed on HBr-treated plants was significantly lower than that of the control treatment ($F = 28.823$; df = 1, 34; $P < 0.001$) (**Figure 3a**). When we analyzed the population dynamics of *D. citri* by developmental instar/stage, these differences persisted across all cases. The number of eggs laid was higher in the control group compared to the HBr-treated plants ($F = 14.794$; df = 1, 34; $P = 0.001$) (**Figure 3b**). Accordingly, the number of young nymphs (N_1, N_2, and N_3) and the number of mature nymphs (N_4 and N_5) was higher in the control treatment than in the HBr-treated plants [$F = 7.101$; df = 1, 16; $P = 0.012$ and $F = 5.703$; df = 1, 25; $P = 0.025$, respectively) (**Figure 3 c,d**). Additionally, the resulting number of adults was also more significant in the control treatment compared to the HBr-treated group ($F = 9.701$; df = 1, 36; $P = 0.007$) (**Figure 3e**). The structure of the sampled population data allowed us to estimate the mortality rates for each stage transition of *D. citri*, enabling us to observe that the mortality of *D. citri*, including eggs, nymphs, and total (from egg to adult), was higher in the HBr-treated plants than in the control plants (**Table 1**).

In addition to observing the psyllid's performance, we studied how this treatment affected the plants' immune system by analyzing the expression of salicylic acid, jasmonic acid, and an antimicrobial peptide (SAMP) markers 48 hours after the first application of HBr. In response to HBr treatment, differential regulation of genes was observed in both the salicylic acid and jasmonic acid metabolic pathways, along with the SAMP marker, with higher expression in the HBr-treated plants. In the salicylic acid pathway, the upstream genes *ICS* (Isochorismate Synthase) and *PAL* (Phenylalanine Ammonia-Lyase) showed significant increases in their expression ($t = 3.295$, df = 1, 7, $P = 0.006$ and $t = 4.276$, df = 1, 7, $P = 0.009$, respectively), indicating early activation of this pathway. Simultaneously, there was a notable surge in the expression of the downstream gene NPR1 (Nonexpressor of Pathogenesis-Related Genes 1) (t = 5.167, df = 1, 7, P = 0.001), indicative of a robust activation of associated defense mechanisms. In the jasmonic acid pathway, the upstream genes *AOS* (Allene Oxide Synthase) and *LOX2* (Lipoxygenase 2) increased their expression ($t = 3.009$, df = 1, 7, $P = 0.010$ and $t = 2.472$, df = 1, 7, $P = 0.021$, respectively), while the downstream genes *MYC2* (Transcription Factor MYC2) and *JAR1* (Jasmonate Resistant 1) also showed significant increases ($t = 4.072$, df = 1, 7, $P = 0.024$ and $t = 2.917$, df = 1, 7, $P = 0.011$, respectively), indicating pathway activation and modulation of the plant's response. Lastly, the SAMP peptide marker reflected an increase in its expression ($t = 2.014$; df = 1, 7; $P = 0.042$) induced by HBr treatment (**Figure 4)**.

Table 1. Mortality rates of *D. citri* at different developmental stages on control and HBr-treated plants, along with corresponding statistical analysis (*t*-test; P < 0.05).

Mortality	Control plants	HBr-treated plants	Statistics
Egg	49.9 ± 16.3	65.0 ± 10.2	$t_{1,7} = 0.817$; $P = 0.441$
Nymphal	35.9 ± 18.2	68.6 ± 11.2	$t_{1,7} = 1.600$; $P = 0.158$
Egg-adult	76.6 ± 5.33	85.7 ± 6.8	$t_{1,7} = 1.008$; $P = 0.347$

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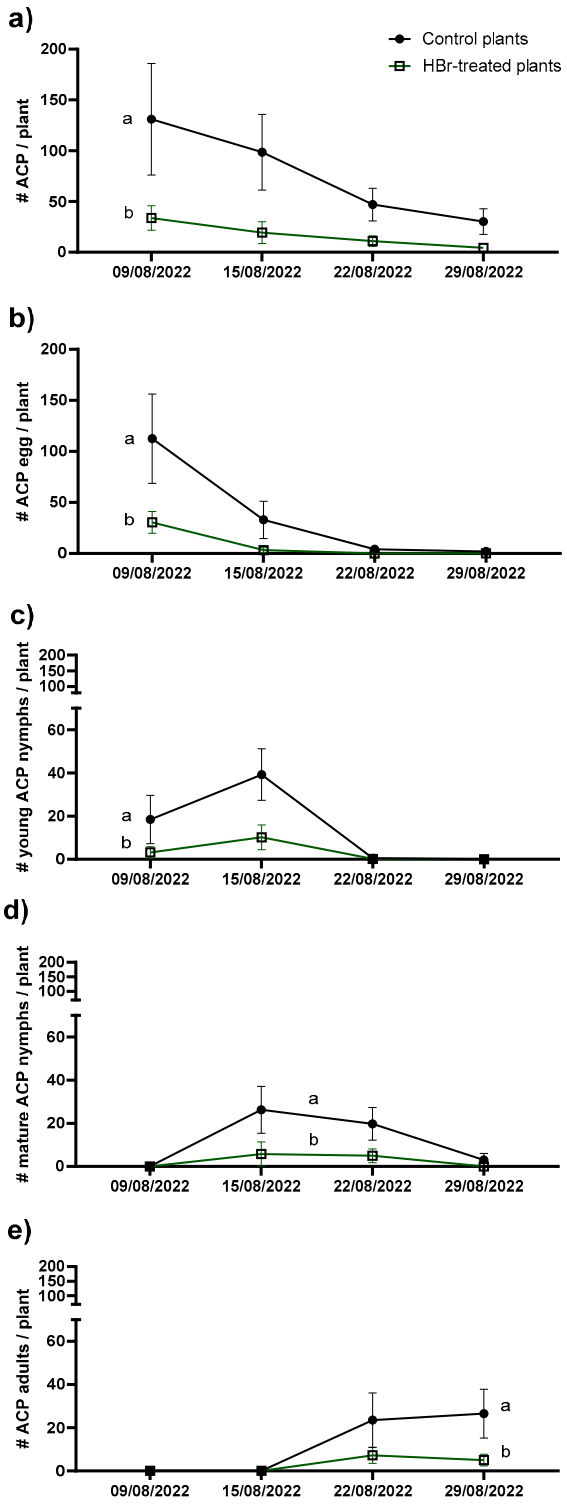

Figure 3. Number of total D. citri (ACP) (eggs + nymphs + adults) (**a**), number of eggs (**b**), number of young nymphs (**c**), number of mature nymphs (**d**), and number of adults (**e**) per plant per week (mean ± SE) for HBr-treated plants and control plants. Different letters between treatments indicate significant differences (GLMM, Repeated Measures; $P < 0.05$).

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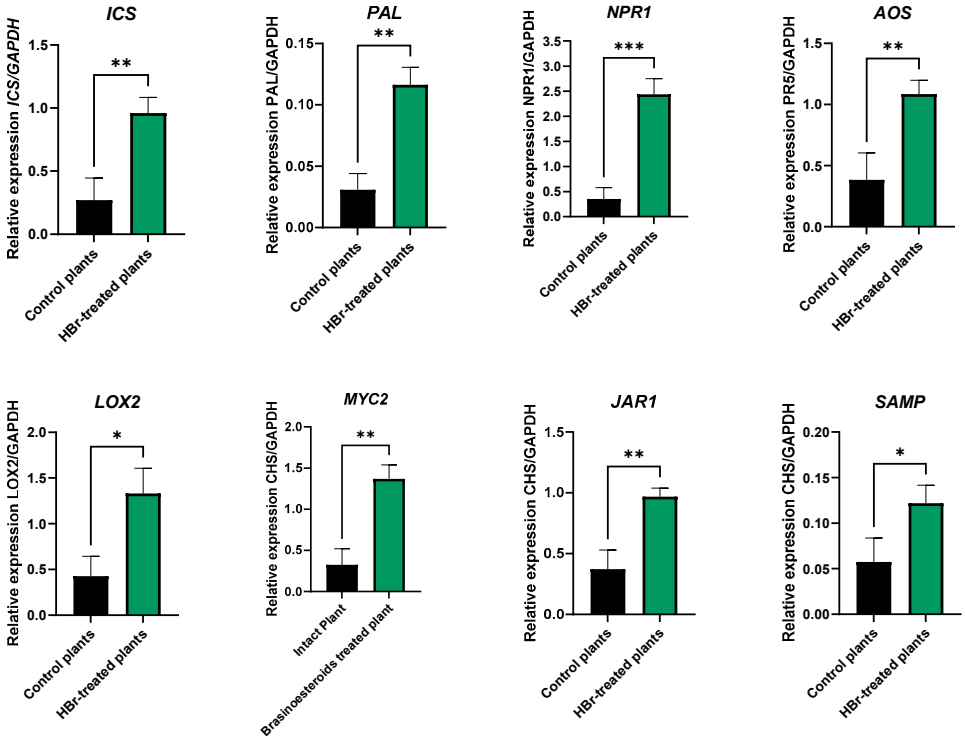

Figure 4. Transcriptional response of the plant defense marker genes ICS (Isochoristame Synthase; from the upstream SA biosynthetic pathway), PAL (Phenylalanine Ammonia-Lyase; from the upstream SA biosynthetic pathway), NPR1 (Nonexpressor of Pathogenesis-Related Genes 1; from the downstream SA biosynthetic pathway), AOS (Allene Oxide Synthase; from the upstream JA biosynthetic pathway), LOX2 (Lipoxygenase 2; from the upstream JA biosynthetic pathway), MYC2 (Transcription Factor MYC2; from the downstream JA biosynthetic pathway), JAR1 (Jasmonate Resistant 1; from the downstream JA biosynthetic pathway) and SAMP (Small Anti Microbial Peptide marker), HBr-treated plants and control plants. Data are presented as the mean independent transcript expression analyses relative to the constitutive GAPDH gene ± SE. *, ** and *** indicate significant differences at the 5%, 1% and 0.01% by t-test.

3. Discussion

Our results provide evidence that treatment with homobrassinolides (HBr) has an impact on reducing the incidence of Huanglongbing (HLB) in newly planted citrus under high psyllid pressure and endemic HLB conditions in Florida. This study adds to the growing body of knowledge about the positive physiological effects of brassinosteroids, such as the advancement of flowering, acceleration of fruit maturation, and increased fruit yield [24–26]. Furthermore, our work aligns with previous studies that demonstrated the capacity of other brassinosteroids to enhance the health of citrus affected by HLB through the activation of plant defense pathways [13].

In our greenhouse experiment, HBr treatment induced differential regulation of genes in the SA and JA metabolic pathways and increased the expression of an antimicrobial peptide marker (SAMP). The coordination between the salicylic and jasmonic acid pathways and the induction of SAMP illustrates a sophisticated mechanism by which HBr may confer citrus plants with increased capacity to resist or delay HLB progression.

It has been suggested that Brs enhance SA-mediated defense responses, and it has been proposed that an adequately sized endogenous Brs pool should be maintained to support SA responses and downstream signaling [22]. Our data strongly support this finding, as exogenous Brs treatment of young citrus plants induced both pathways leading to SA biosynthesis, the chorismate mutase and

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the isochorismate synthase pathways, and also induced downstream immune-related gene expression.

The early activation of the salicylic acid pathways, as indicated by a significant increase in the expression of *ICS* and *PAL* genes, suggests that HBr treatment could prime plants for a rapid and effective response against pathogens. This early activation is crucial, as salicylic acid plays a central role in acquired systemic resistance [27], allowing the plant to bolster its defenses before the pathogen can establish itself. Furthermore, the notable increase in *NPR1* expression, a key regulator of salicylic acid-mediated gene expression, underscores the potent activation of related defense mechanisms, potentially explaining the delay in HLB progression in HBr-treated plants. *NPR1* is crucial in controlling HLB due to its role in regulating the immune balance of plants [28–31]. CLas infection triggers unbalanced immune responses, leading to an overaccumulation of callose and reactive oxygen species (ROS), which causes phloem obstruction and the development of HLB symptoms [32]. However, it has been discovered that overexpression of the *AtNPR1* gene from *Arabidopsis thaliana* in susceptible varieties confers robust HLB tolerance [33]. The overexpression of *AtNPR1* suppresses the overaccumulation of callose and ROS induced by CLas in citrus and *Arabidopsis*, respectively [34]. The function of *NPR1* is centered on its interaction with SA, where *NPR1* acts as an SA receptor, promoting redox changes that convert *NPR1* from an oligomeric complex to monomers that move to the nucleus. There, *NPR1* interacts with TGA transcription factors to activate the transcription of defense genes, including *PR* genes [32]. Sarkar et al. [32] suggest that *NPR1* overexpression is a promising route for the development of HLB-tolerant citrus through genetic manipulation. In our work, we have demonstrated that plants treated with HBr significantly overexpress the *NPR1* gene.

Concurrently, activation of the jasmonic acid pathway, evidenced by increased expression of *AOS* and *LOX2* genes, along with significant increases in *MYC2* and *JAR1*, reflects modulation of another critical pathway in the plant's response to stress and pathogen attack. This pathway, complementary to the salicylic acid pathway, is associated with defense against herbivores and certain types of microbial infections [35,36], suggesting an integrated defense strategy that could limit the effectiveness of HLB vectors, as observed in the greenhouse experiment. The *AOS* and *LOX2* genes play essential roles in the initial stages of JA biosynthesis. At the same time, *MYC2* and *JAR1* are fundamental in the signaling and response to JA, modulating the expression of defensive genes and resistance to herbivores [35]. The observed overexpression of *AOS* and *LOX2* indicates an activation of JA metabolism in response to HBr treatment, suggesting increased JA production. *MYC2*, a key transcription factor in the JA pathway, and *JAR1*, involved in the conjugation of JA with isoleucine to form the active JA-Ile complex, also showed increased expression. This confirms the pathway's activation and suggests a refined modulation of the plant's defensive response upon HBr treatment. This activation of the JA pathway could explain the relationship between HBr treatment and increased *D. citri* mortality in HBr-treated plants. The overexpression of these genes indicates that plants are potentially amplifying their arsenal of defensive responses, which include the production of toxic or repellent secondary metabolites for herbivores, the induction of defense-related proteins such as protease inhibitors, and the fortification of physical structures of the plant [37]. The results obtained in the greenhouse experiment can explain the observed decrease in populations of *D. citri* and the damage caused by the CRM on HBr-treated plants in the field experiment. Previous research demonstrates the connection between JA signaling and herbivore resistance, showing that increased protease activity induced by JA can reduce the incidence of mites and other herbivores on plants [38–43]. These proteases interfere with protein digestion in herbivores, limiting their growth and survival in the host plant [44,45]. Therefore, HBr treatment appears to exert a systemic effect on plants, inducing JA-mediated defense responses resulting in reduced susceptibility to herbivore attacks. Although the quantity of CREM was similar in both treatments, the surface affected by these mites on the fruit was significantly smaller in HBr-treated plants, suggesting that while the treatment did not directly affect the mite population, it did improve the plant's ability to mitigate damage caused by them. This protective effect could be related to the induction of specific proteins or changes in the plant surface composition that hinder mite feeding or

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reproduction. The lower quantity of ACP on tender shoots of HBr-treated plants also highlights the treatment's impact on reducing herbivore viability.

The increase in SAMP peptide expression underscores a broad response to stress induced by HBr treatment, suggesting that HBr activates specific defense pathways and enhances the plant's ability to handle stress, potentially improving its overall resilience to various threats. This peptide was described by Huang et al. [43] with potent antimicrobial activity that directly targets CLas but also activates innate immunity in citrus trees, offering both therapeutic and preventative capabilities. In our case, the HBr treatment increased its content in the HBr-treated plants.

The finding that HBr delays HLB progression in young trees opens up an exciting possibility in areas where HLB is endemic, such as Florida. Currently, citrus growers in Florida are increasingly using psyllid exclusion methods, such as Individual Protective Covers (IPCs). These are polyethylene mesh bags with pores smaller than the size of the psyllid's body, effectively excluding the insect vector. The effectiveness of this tool in maintaining newly planted citrus trees free from disease has been recently reported [47,48]. IPCs have proven to be a promising strategy for protecting young trees from HLB, keeping them symptom-free and negative for HLB in trials. Trees under IPCs show faster growth and higher chlorophyll accumulation, suggesting that this approach could be helpful in extending tree productivity and improving the health of infected ones. However, eventually, IPCs have to be removed due to tree growth (typically 2 or 3 years after planting), leaving young trees exposed to infection. Any treatment that could prolong tree health is highly desirable in this scenario. We are currently investigating the effectiveness of a system that combines a physical barrier (the IPCs) followed by treatments with HBr.

In conclusion, HBr treatment represents a promising strategy for HLB management in citrus, offering an additional tool in the arsenal against this disease. The role of brassinosteroids, specifically HBr, in modulating plant immune response to biotic stress is becoming increasingly evident. However, much remains to be elucidated. Future research should focus on understanding more thoroughly the underlying molecular mechanisms of HBr's observed effects and optimizing dosage and application timings to maximize benefits under commercial conditions. Additionally, it would be interesting to explore the interaction between HBr and other HLB management methods, such as the aforementioned IPCs, integrated management of ACP, and other citrus pests, and their effect on different citrus varieties, to develop more holistic and sustainable management approaches.

4. Materials and Methods

4.1. Field Experiment

4.1.1. Plant Material, Experimental Design, and Treatments

Six replicated plots of three 18-month-old 'Valencia' trees on 'Cleopatra' rootstock per treatment in a randomized complete block design were planted at the Southwest Florida Research and Education Center in Collier County, Florida (26.466966 N, 81.446917 W). Trees were fertilized using conventional granular fertilizer (8N-4P-8K; Diamond R, Fort Pierce, FL). Irrigation was by under-tree microjets. No insect management was performed in the experimental block during the experiment. Treatments were water (control) and 1 μM HBr (Homobrassinolide 0.1%, Repar Corp, Maryland, USA), and were performed monthly for 1 year.

4.1.2. Candidatus Liberibacter asiaticus (CLas) Detection

For bacterial infection estimation, leaves were collected from each individual tree every 3 months. Detection was performed as in [48]. Briefly, three to four mature leaves from recent flushes were randomly collected from the middle tree of each replicated plot every 3 months for 1 year. Petioles and midribs of leaves were excised, minced with a razor blade, lyophilized in a FreeZone 6 freeze-dry system (Labconco, Kansas City, MO, USA), and pulverized using a mini bead beater (Biospec products, INC. OK, USA). According to the manufacturer's instructions, DNA from 100 mg of pulverized leaves was extracted using the Wizard Magnetic 96 DNA Plant System (Promega

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Corporation, Madison, WI, USA). DNA was quantified using a microplate reader (Synergy HTX Multimode Reader, Biotek instruments, INC, VT, USA) and normalized to 10 ng/µl. CLas was detected by quantitative real-time polymerase chain reaction (qRT-PCR) using a 7500 Fast Real-Time PCR system (Applied Biosystem, Foster City, CA, USA). The primers and probes used for detection were HLBas and HLBr and probe HLBp, respectively, as described in [49]. Samples with a Ct-value of less than 36 were considered as CLas-positive.

4.1.4. CRM and ACP Samplings

Trees set fruit for the first time 1 year after planting. We estimated the damage caused by rust mites and their number per fruit in these fruits. For this purpose, we counted in 8 randomly selected fruits on each central tree of each repetition the number of mites present in 1 cm^2 of fruit surface using a 15x magnifying lens. Two measurements were taken per fruit. Additionally, the scarred surface caused by CRM damage was estimated for each fruit [50]. In each of the trees, the population of adult D. citri present was estimated using two stem-tap samples [51], and from two randomly selected shoots with tender leaves, the number of eggs, nymphs, and adult D. citri present was counted.

4.2. Greenhouse Experiment

4.2.1. Plants and Insects

Two-year-old "Valencia" cultivar plants, grafted onto rootstock "X-639," a hybrid cross of Cleopatra mandarin [*Citrus reshni* (Tanaka)] and *Poncirus trifoliata* (L) Raf., were used. These citrus plants were obtained directly from a certified nursery. They were left undisturbed for two months without receiving any chemical treatment in an isolated and well-protected greenhouse before being used in the experiments. Three weeks before the start of the experiments, the plants were defoliated entirely and pruned, leaving 4-5 lateral branches on each one, with approximately 4-5 buds. They were allowed to re-sprout in the greenhouse where the experiment was conducted, each inside its corresponding cage. After three weeks, all the plants had an average of feather shoots of 7.2 ± 0.8, the preferred state for ACP oviposition [52].

Adults of D. citri were obtained from colonies established at the Southwest Florida Research & Education Center (SWFREC). These colonies were reared on Murraya paniculata (L.) Jack plants, with initial specimens collected from experimental fields at SWFREC. A binocular stereo microscope was used to isolate the psyllids and distinguish between females and males.

4.2.2. Diaphorina Citri Performance on HBr-Treated Plants

The experiment was conducted in a 20 x 6 m greenhouse located at SWFREC. The greenhouse was accessed through a double door. One Datalogger (HOBO U23 Pro v2 External Temperature/Relative Humidity Data Logger, HOBO dataloggers, Bourne, MA, USA) was placed in the center of the greenhouse to record temperature and relative humidity. This experiment was conducted in August 2022 and the environmental conditions were: average night/day temperature in the greenhouse: 34/20 °C; relative humidity varied between 45% and 96%. Natural photoperiods were about 11 h.

Five replicates were conducted for each treatment to be tested. The two treatments included water (control) and 1 µM HBr (Homobrassinolide 0.1%, Repar Corp, Maryland, USA), which were directly sprayed onto the plants 48 hours before releasing the *D. citri* adults and then weekly. Each replicate involved introducing a plant with receptive flushes for *D. citri* oviposition into an individual entomological cage measuring 60 × 60 × 90 cm (Insect Rearing Cage, Entomological Livestock Supplier, Parma, Italy), with five pairs of *D. citri* carefully selected and placed on the plants. Plants were watered twice a week. Four weekly evaluations assessed the numbers of *D. citri* (adults, eggs, and nymphs) per plant.

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4.2.3. RNA Extraction and qRT PCR Analysis of Gene Expression

For gene expression determination, apical parts from five control and five treated plants were collected 48 hours after HBr treatment. Each sample was crushed in liquid nitrogen using a mortar and pestle. Total RNAs from 0.1 g of fresh leaf tissue was extracted with the RNeasy Plant Mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The RNA was quantified, and its purity was confirmed using a nanodrop. Following the manufacturer's instructions, one µg of total RNA was used for gDNA removal and cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). The cDNA samples were stored at -80°C to further assay the defense genes' expression in citrus.

Real-time PCR amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using NZYSupreme qPCR Green Master Mix (2x) (NZYTech, Lisboa, Portugal). Reactions were performed in a 10 µl volume containing 0.5 µM of each primer and 1 µg of cDNA template. The cycling program was set to 2 min of the pre-cycling stage (95 °C), 40 cycles of 5 s at 95 °C, 25 s at 58 °C and 15 s at 72 °C. Primer sequences of defensive genes ICS, PAL, NPR1, AOS, LOX2, MYC2, JAR1, SAMP, and the housekeeping gene *GADPH* used as a standard control gene for normalization are represented in **Error! Reference source not found.**).

4.3. Statistical Analysis

The number of preyed *E. kuehniella* eggs by males and females of *N. tenuis*, necrotic rings, and wilted during the zoophagy and phytophagy trial were subjected to a *t*-test ($P < 0.05$). Likewise, the duration of the biological cycle of both tested strains from egg to adult, for both males and females, was also subjected to a *t*-test ($P < 0.05$). The progeny and the longevity of the females were also analyzed using a *t*-test ($P < 0.05$). The greenhouse experiment aimed to assess the impact of HBr treatments on *D. citri* performance by studying various variables. A Generalized Linear Mixed Model (GLMM) was employed to analyze the total number of ACP, eggs, young, and mature instars, allowing for repeated measures. We utilized a Poisson distribution and a logarithmic link function to detect differences between the HBr-treated plants and the control group. Treatment was considered a fixed factor, while the week was treated as a random one. To explore variations among treatment levels, a post hoc analysis was conducted using the LSD test ($P < 0.05$). Dates with zero values (such as nymphs in the first week) were excluded from the statistical analysis. Mortality rates in both treatments were compared using a t-test ($P < 0.05$).

Table 2. List of genes and primers used to analyze the expression of defense-associated genes in citrus.

Gene[a]	Gene name	Citrus ID	Primer Sequence (5′→3′)
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase,*	LOC102624117	**FW:** GGAAGGTCAAGATCGGAATCAA **RV:** CGTCCCTCTGCAAGATGACTCT
PAL	*Phenylalanine ammonia-lyase-like*	LOC102620464	**FW:** CACATTCTTGGTAGCGCTTTG **RV:** AGCTACTTGGCTGACAGTATTC
ICS	*Isochorismate synthase 2, Chloroplastic*	LOC102630235	**FW:** GGAGGAGGAGAGAGTGAATTTG **RV:** GGGTTGCTTCCTTCTACTATCC
NPR1	*BTB/POZ domain and ankyrin repeat-containing protein*	LOC102617188	**FW:** GTACCTTGAAAACAGAGTTGGACTGG **RV:** TGCTCCTCTTGCATTTTGAAAGGTG
MYC2	*Transcription factor MYC2*	LOC102626457	**FW:** TGCATCTACAGCCGACCC **RV:** TAGGTCCAGCCCTCACGA
LOX2	*Linoleate 13S-lipoxygenase 2-1, chloroplastic-like*	LOC102629656	**FW:** GAACCATATTGCCACTTTCG **RV:** CGTCATCAATGACTTGACCA
AOS	*Allene oxide synthase*	AY243478	**FW:** AGATCTTATTCCCGAACATGGT **RV:** CGGACTTCATCAACGGCAT
JAR1	*Jasmonate Resistant 1*		**FW:** AAGGCGATGCAGTCACAATG

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		LOC102611440	**RV:** TGGTGGAAATCAGGACCAAAG
SAMP	*response A/B barrel domain-containing protein HS1*	LOC102628374	**FW:** AACAGGGGCAAGAATGTGAGCAT **RV:** ACACGTACTGTTGTCGGTTTGTAGTCA

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