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Effect of Feeding Stage and Density of Whiteflies on Subsequent Aphid Performance on Tobacco Plants

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Abstract: *Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1) is a cosmopolitan, highly polyphagous agricultural pest, which has the capacity to displace other native insect herbivores. Here, equipped with an integrated approach, the effect of developmental stages and feeding density of whiteflies on *Myzus persicae* performance in tobacco plants are investigated. Bioassay results showed that *B. tabaci* nymphs, but not adult, pre-infestation significantly reduced survival and fecundity of *M. persicae*, and the strongest resistance to *M. persicae* was detected at the medium density (9–10 nymphs/cm^2). Neither low nor high feeding density of *B. tabaci* nymphs triggered visible resistance to aphids. However, no significant results were detected in salicylate-deficient *NahG* plants after *B. tabaci* nymph infestation. In addition to performance distinctions, hormone quantification and qPCR results revealed very different effects for nymph and adult whitefly stages on the defense responses in tobacco. *B. tabaci* nymph infestation significantly increased SA accumulation and SA-responsive genes (*PR-1a*, *PR-2a*) expression but suppressed JA-regulated responses. In contrast, tobacco plants responded to adult infestation by slightly increasing in both SA- and JA-regulated defenses. Furthermore, higher transcription level of *Bt56*, coding gene of a secretory salivary effector, was recorded in nymphs vs. adults, while silencing of *Bt56* by virus-induced gene silencing (VIGS) partly impaired the aphid resistance induced by *B. tabaci* nymphs. These results proved that the induction of tobacco defense responses varied with the feeding stages of whiteflies: nymphs of *B. tabaci*, but not adults, induced a defense response against aphids, with a density threshold for this induced resistance.

Keywords: *Bemisia tabaci* MEAM1; nymph; adult; infestation levels; *Myzus persicae*; induced defense

Citation: Li, Y.; Qu, C.; Yan, X.; Sun, X.; Yin, Z.; Zhao, H. Effect of Feeding Stage and Density of Whiteflies on Subsequent Aphid Performance on Tobacco Plants. *Agronomy* **2022**, *12*, 1025. https://doi.org/10.3390/agronomy12051025

Academic Editors: Francesco Calzarano and Muxing Liu

Received: 25 March 2022
Accepted: 21 April 2022
Published: 24 April 2022

1. Introduction

Co-evolving with herbivores over millions of years, plants derived complex and sophisticated defense systems to defeat the stress from herbivores, which include constitutive and induced defenses [1]. Inducible defenses refer to physiological and biochemical changes in plants in response to stimuli of damage from consumers, which can negatively influence the feeding, development, and reproduction of attacking herbivores with the production of toxic metabolites or changes in the quality and quantity of tissue composition [2,3]. The induction of defense responses is orchestrated by plant hormones, of which salicylic acid (SA) and jasmonic acid (JA) are the major players [4,5]. Induced defenses may be broad in spectrum or may target a specific pest species or biotype, which is usually affected by many factors, including feeding type, duration, saliva components, and even level of infestation [6,7].

Phytophagous arthropods that are in the same niche can cause different reactions in plants, as plants can accurately perceive biotic attackers and activate appropriate defense responses [8–10]. In squash, whitefly-induced defense response varied with biotypes [11], which is also observed with aphid *Sitobion avenae* and *Tetranychus urticae* mite on tomato

plants [7,12]. Even different feeding stages of an insect herbivore present diverse stimuli to host plants. For example, the physiological responses of pumpkin and gene expression in tomato were different depending on whether infested by *Bemisia tabaci* adults or nymphs [13]. Differences in host reactions induced by different biotypes, and developmental stages of phloem feeders, may result from different saliva components.

Herbivore-induced defense in plants can also be density-dependent [14–16]. In *Arabidopsis*, the intensity of defensive reactions triggered by aphids varied with the feeding density [17]. Shiojiri et al. (2010) found a positive correlation between the amount of volatile release and *Pieris rapae* feeding density, within limits, on cabbage plants [15]. Furthermore, feeding densities may affect the plant-mediated interaction between herbivores. For instance, Kroes et al. (2017) showed that plant-mediated interactions between *Brevicoryne brassicae* aphids and *Plutella xylostella* caterpillars are density dependent [18], and behavioral choices of the parasitoid *Cotesia glomerata* towards herbivore-induced plant volatiles were dependent on the aphid density infesting the plant [19].

Induced defenses have been proved to play crucial roles in the plant-mediated interaction between herbivores [20]. *Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1) is an exotic pest insect, which is highly adaptable and has displaced many native species [21,22]. Increasing evidence has found that the induced plant resistance mediated by *B. tabaci*, along with other biological characteristics, e.g., higher fecundity, pesticide resistance, and climate adaptability, were closely involved in this type of interspecific competition [23,24]. For instance, by triggering induced defense responses, silver leaf whitefly infestation inhibited the growth and feeding of cabbage looper, resulting in decreased oviposition, larval survival of *Liriomyza trifolii*, and decreased performance of *Trialeurodes vaporariorum* on their host plants [25]. Research has shown that *B. tabaci* biotype A at different developmental stages causes various transcription reprogramming in tomato plants [26], and indirect plant defenses induced by *B. tabaci* are density-dependent in Lima bean [27]. Differences in host reactions caused by different biotypes and developmental stages of phloem feeders may result from different variety and quantity of the saliva components [28]. Although important roles of *B. tabaci* salivary effectors, such as *Bt56* (an SA defense elicitor), in regulating plant defenses have been proved [29], no relevant reports have compared the saliva of whitefly adults and nymphs, and what is known is that *B. tabaci* nymphs had lower alkaline phosphatase activities than adults [30]. As differences in plant responses may contribute to the competitive displacement among insect herbivores, whether the plant quality changes mediated by variety feeding stages and densities of *B. tabaci* cause the same effect on subsequent herbivores remains unknown.

Myzus persicae, another common sap-sucking pest, also has a broad host distribution which can co-occur with whitefly on the same plant. Both these species are important pests on vegetables, field crops, and ornamental plants, and they cause severe economic losses by direct feeding and particularly by viruses transmitting [17,28]. Our preliminary experiment showed that *B. tabaci* has the capacity to displace *M. persicae*, as they occupy the same ecological niche. In this study, a plant–whitefly–aphid model was created. The *M. persicae* performance, activity of defense signaling on tobacco plants infested by *B. tabaci* MEAM1 nymphs and adults at different densities, and difference in salivary effector between *B. tabaci* MEAM1 nymphs and adults were investigated using bioassay, enzyme-linked immunosorbent assays, quantitative real-time PCR, and virus-induced gene silencing techniques combined with the introduction of transgenic *NahG* (salicylate-deficient) tobacco plants. The objective of this investigation was to answer the following questions: (1) Does *B. tabaci* adult and nymph pre-infestation result in similar effects on subsequent *M. persicae* on tobacco plants? (2) Do changes in *B. tabaci* density alter the performance of subsequent *M. persicae*? (3) What was the cause of differences in *M. persicae* performance? Investigating effects of insect feeding stage and density on plant defense responses can provide novel insights in plant-mediated interactions between multiple attacking insects and promote the development of novel strategies for controlling these pests.

2. Materials and Methods

2.1. Plants and Insect

Tobacco (*Nicotiana tabacum* L.) *Xanthi-nc* WT (wild type) and *NahG* (suppresses SA accumulation) seeds were obtained from Nanjing Agricultural University, Institute of Plant Protection (Nanjing, Jiangsu Province, China). Seeds were sown in plastic trays (50 cm × 25 cm) and maintained under standard greenhouse conditions (23 ± 2 °C, 75 ± 5% RH). Plants at the two-to-four-leaf stage were individually transplanted into plastic pots (10 cm depth, 12 cm diameter) and placed in insect-proof, screened cages (50 cm × 50 cm × 50 cm; 50 meshes). The plants were watered as necessary and fertilized every 2 weeks at a rate of 0.05 g (N:P:K = 20:20:20) per plant.

Bemisia tabaci Middle East-Asia Minor 1 (MEAM1) were originally collected from cabbage plants, and *Myzus persicae* were obtained from tobacco plants in Taian, Shandong Province, China. Colonies of both species were maintained on tobacco in the greenhouse for more than 30 generations. *B. tabaci* was identified based on the mitochondrial DNA COI gene sequence.

All bioassay experiments were conducted in an artificial climate chamber (RTOP-D model; Top Instrument Corporation, Zhejiang, China) at 23 ± 2 °C and 75 ± 5% RH, with a photoperiod of 12:12 (L:D) h.

2.2. Plants Pre-Infested with Whitefly Nymphs

For this method, see reference Xue et al. (2010) [31]. Plants at the five-leaf stage were placed in individual nylon screen cages (50 cm × 50 cm × 50 cm) with approximately 100 (±10), 300 (±10), 500 (±10), or 700 (±10) whitefly adults (1:1 female/male) per plant. After 4 h of feeding, adult whiteflies were removed by aspiration to synchronize egg hatching and nymphal development. We selected plants with 1–2 nymphs/cm^2 (N1), 5–6 nymphs/cm^2 (N2), 9–10 nymph/cm^2 (N3), and 13–14 nymph/cm^2 (N4) as treatments. Plants without whiteflies were used as controls in screened cages in the same greenhouse.

2.3. Plants Pre-Infested with Whitefly Adults

Infestation with *B. tabaci* adults was performed using the procedures of Zhang et al. (2015) [32]. Pyriproxyfen (600-fold diluted, 10% active ingredients, emulsifiable concentrate), which interferes with egg development of *B. tabaci* [33], was smeared evenly on the third and fourth leaves of ten-leaf-stage vigorous tobacco plants. These treated plants were enclosed in fine-mesh nylon cages (15 cm × 12 cm × 4 cm; 80 meshes). Approximately 50 (±2) (A1), 100 (±5) (A2), 200 (±10) (A3), or 400 (±20) (A4) whitefly adults (1:1 female/male) per leaf were collected with a suction sampler and released inside each cage [34]. Control plants without whiteflies were smeared and encased similarly.

2.4. Effects of Whitefly Adult and Nymph Pre-Infested Tobacco Plants on the Performance of Aphids

Whitefly adult-infested plants were used 1 day after infestation, and the infested fourth and uninfested seventh leaves were considered as local and systemic leaves, respectively. Whitefly nymph-infested plants were used 20 days (third instar) after infestation, and the infested fourth leaves and uninfested seventh leaves served as local and systemic leaves, respectively.

Fifteen mature, apterous *M. persicae* were placed on the lower surfaces of systemic leaves of pre-infested and uninfested plants and confined using a clip-on leaf cage (2 cm depth, 4 cm diameter). Aphids were allowed to reproduce for 24 h and then removed using a small soft brush, and 20 newborn *M. persicae* nymphs were caged on the same leaf. Survival of the young aphids was recorded daily for 8 days until they matured. After 8 days, all but two adults were removed; the two adults were confined in a leaf clip-on cage on the lower surface of each test leaf for fecundity determination. Fecundity was calculated by counting the number of offspring of each adult until it died; after daily counting, newborn nymphs were carefully removed with a fine brush to avoid mechanical damage to leaf surfaces. Each treatment had six replications.

2.5. Qualification of Endogenous Phytohormones

Experimental WT tobacco plants were obtained as described above. The systemic leaves from whitefly adult- and nymph-infested plants (with uninfested plants as controls) were sampled 1 and 20 days after infestation, respectively. SA and JA content were determined using an enzyme-linked immunosorbent assay (ELISA) according to Yang et al. (2001) [35]. The antibodies used in the ELISA test were supplied by the Phytohormones Research Institute (China Agricultural University, Beijing, China). In brief, 0.2 g fresh sample leaves were extracted for 24 h at 4 °C and then purified by passing through C_{18} Sep-Pak cartridges (Thermo, USA). Microtitration plates were coated with 50 µL sample and 50 µL antigen (0.25 µg mL^{-1}) against the hormones. The coated plates were then incubated for 45 min at 37 °C. Next, each well was filled with 100 µL antibodies (20 µg mL^{-1}) and incubated for another 1 h at 37 °C. Finally, 100 µL color-appearing solution containing 0.008% (v) H_2O_2 and 2 mg mL^{-1} O-phenylenediamine (OPD) was added to each well. Plates were incubated for 15 min at 37 °C in the dark, and reactions were subsequently terminated using 50 µL 2 M H_2SO_4 per well. The absorbance was recorded at 490 nm. Each concentration was analyzed in triplicate in each of three biologically independent treatments.

2.6. Virus-Induced Gene Silencing

Bt56 (Genbank accession no. KY986870.1)-silencing vector construction method was performed according to Luan et al. (2013) [24]. Extraction and reverse transcription of RNA were performed as described above. The target fragment was amplified and cloned into the pBIN2mDNA1 (*BamHI-XbaI*-digested) plasmid to construct the silencing-vector (pBIN2mDNA1-Bt56). Then, the vector was transformed into *Agrobacterium tumefaciens* EHA105 by electroporation before mixing *A. tumefaciens* cultures (silencing-vector) with equal volumes of *TbCSV* (a helper virus) and infiltrating the mixtures (ABS600 = 0.6) into the stem of each plant (four-leaf stage). Plants inoculated with TbCSV and *A. tumefaciens* (empty vector) were treated as control plants. The second leaf from the top of the gene-silenced plants (five-true-leaf stage) was sampled for detection of silencing fragments using qRT-PCR. Next, Bt56 expression in *B. tabaci* nymphs that had been feeding on the pBIN2mDNA1-*Bt56*-injected tobacco seedlings for 15 days was assessed by qPCR. Silenced and control plants were used for the aphid bioassay (as described above).

2.7. Quantitative Real-Time PCR

For gene expression analyses of tobacco defense genes, experimental tobacco plants were obtained as described above. The systemic leaves from infested and uninfested tobacco plants were sampled for analysis of defense gene expression. For gene expression, analyses of whitefly *Bt56* genes, the head of *B. tabaci* at different developmental stages (nymphs, and adults, female/male = 1:1), were sampled for analysis of defense gene expression. Total RNA samples were isolated. cDNA was synthesized using the TransScript RT-PCR Kit (Transgen Biotechnology, Beijing, China). *PR-1a*, *PR-2a*, *TPI*, and *PI-II* RNA levels were measured using qRT-PCR. qRT-PCRs analyses were performed on a LightCycler® 96 Real-Time PCR System (Roche Laboratories, Basel City, Switzerland) using qPCR SYBR Green kits. Each gene was analyzed in triplicate in each of three biologically independent treatments. The average threshold cycle (Ct) was calculated per sample. The relative expression levels were calculated with the 2-ΔΔCT method. The reference gene *Actin* (Genebank accession No. X69885.1) and *TAF* (TATA box binding protein associated factor) gene was used for transcript normalization, respectively.

2.8. Statistical Analyses

Data were analyzed using the SPSS statistical software package version 18.0 (SPSS, Chicago, IL, USA). *M. persicae* survival was tested with a Cox proportional hazards model. *M. persicae* fecundity, phytohormone levels, and expression of defense genes were analyzed

using two-way analysis of variance, followed by separation of means using Tukey's tests at a significance level of 5% ($p < 0.05$).

3. Results

3.1. Infestation of Whitefly Nymphs and Adults at Different Densities on the Survival and Fecundity of Subsequent Aphids on WT Plants

Based on the Cox proportional hazards model, feeding stage (F = 8.570; df = 1, 59; $p = 0.005$) and infestation density (F = 9.450; df = 4, 59; $p < 0.001$) had significant effects on *M. persicae* survival in WT plants. Feeding by *B. tabaci* nymphs had a significant effect on aphid survival, except at the N1 (1–2 nymphs/cm^2) density, on WT tobacco plants. The negative influence on aphid survival was first detected at the feeding density N2 (5–6 nymphs/cm^2), and then at the N3 (9–10 nymphs/cm^2) feeding density, aphid survival decreased dramatically and was 50.46% (F = 10.749; df = 4, 29; $p < 0.001$) lower than that in the uninfested control. The inhibition of aphid survival at the N4 density (13–14 nymphs/cm^2) was less than that at N3 and was 11.65% (F = 38.464; df = 4, 29; $p < 0.001$) lower than that in the uninfested control (Figure 1a). No density of the *B. tabaci* adults affected aphid survival on tobacco plants (Figure 1b).

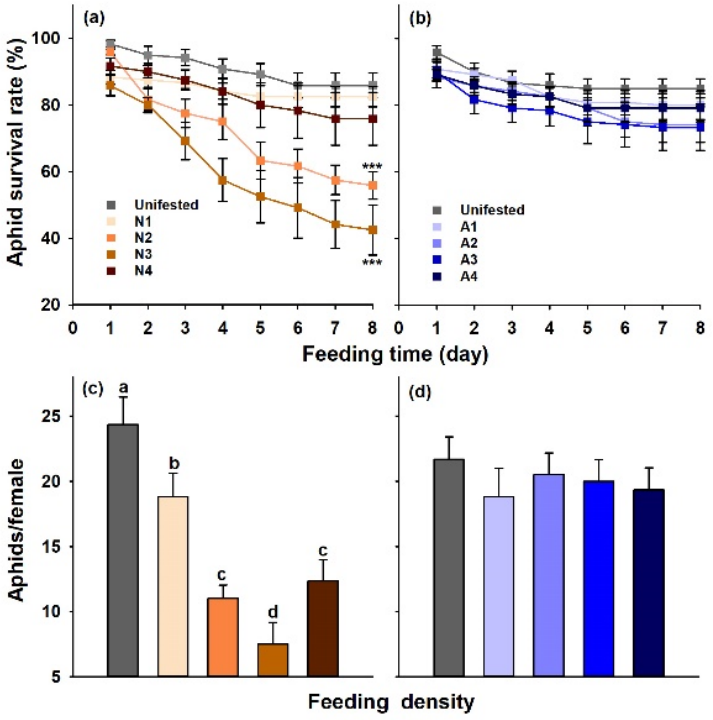

Figure 1. Performance of *M. persicae* on wild-type tobacco plants infested by *B. tabaci* MEAM1. Survival rate of *M. persicae* on tobacco plants infested by *B. tabaci* MEAM1 nymphs (**a**) and adults (**b**) at different densities. Fecundity of *M. persicae* on tobacco plants infested by *B. tabaci* MEAM1 nymphs (**c**) and adults (**d**) at different densities. Letters N1, N2, N3, and N4 indicate infestation level of nymphs at 1–2, 5–6, 9–10, and 13–14 nymphs/cm^2, respectively. Letters A1, A2, A3, and A4 represent adult infestation level of 50 (±2)/leaf, 100 (±5)/leaf, 200 (±10)/leaf, and 400 (±20)/leaf, respectively. Error bars represent standard error of the means. The different letters above the bars indicate values that are significantly different ($p < 0.05$).

Feeding stage (F = 22.962; df = 1, 59; $p < 0.001$) and infestation density (F = 8.594; df = 4, 59; $p < 0.001$) had significant effects on *M. persicae* fecundity in WT plants. After infestation by *B. tabaci* nymphs, aphid fecundity at the N1 (1–2 nymphs/cm^2) density decreased by 22.6% (F = 8.594; df = 4, 49; $p = 0.020$) compared with that in the uninfested control. The negative effect on fecundity of aphids was most obvious at the N3 (9–10 nymphs/cm^2) feeding density, with fecundity 69.18% (F = 8.594; df = 4, 49; $p < 0.001$) lower than that in

the control. At the N4 (13–14 nymphs/cm^2) density, aphid fecundity was 49.31% (F = 8.594; df = 4, 49; $p < 0.001$) lower than that in the uninfested control (Figure 1c). Infestation by *B. tabaci* adults had no significant effect on aphid fecundity at any of the four different levels of infestation (Figure 1d).

3.2. Infestation of Whitefly Nymphs and Adults at Different Densities on the Survival and Fecundity of Subsequent Aphids on NahG Plants

Based on the Cox proportional hazards model, feeding stage (F = 0.285; df = 1, 59; $p = 0.596$) and infestation densities (F = 0.320; df = 4, 59; $p = 0.863$) had no significant effects on *M. persicae* survival in *NahG* plants. No density of the *B. tabaci* adults and nymphs affected aphid survival on *NahG* tobacco plants (Figure 2a,b).

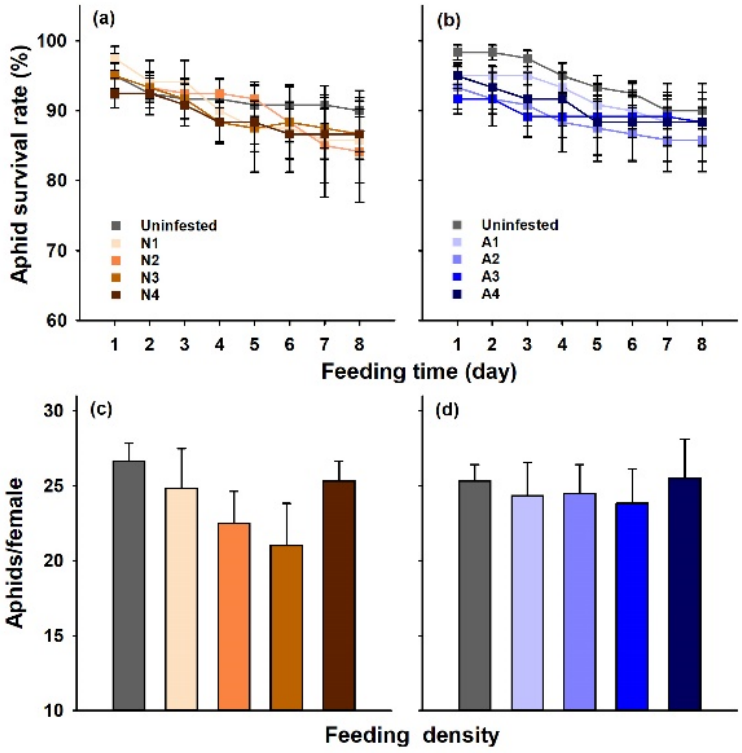

Figure 2. Performance of *M. persicae* on *NahG* plants infested by *B. tabaci* MEAM1. Survival rate of *M. persicae* on tobacco plants infested by *B. tabaci* MEAM1 nymphs (**a**) and adults (**b**) at different densities. Fecundity of *M. persicae* on tobacco plants infested by *B. tabaci* MEAM1 nymphs (**c**) and adults (**d**) at different densities. Error bars represent standard error of the means.

Feeding stage (F = 0.225; df = 1, 59; $p = 0.637$) and infestation density (F = 0.939; df = 4, 59; $p = 0.449$) had no significant effects on *M. persicae* fecundity in *NahG* plants. Neither *B. tabaci* adults nor nymphs feeding had significant effect on aphid fecundity at any of the four different levels of infestation (Figure 2c,d).

3.3. Infestation of Whitefly Nymphs and Adults at Different Densities on the Defense Hormone Levels

Feeding stage (F = 115.539; df = 1, 89; $p < 0.001$) and infestation density (F = 76.570; df = 4, 89; $p < 0.001$) had significant effects on SA levels in tobacco plants. *B. tabaci* nymph infestation significantly increased SA level. SA accumulation was first detected at feeding density N2 (5–6 nymphs/cm^2), and then at the N3 (9–10 nymphs/cm^2) feeding density, SA level increased dramatically and was 2.62-fold (F = 4.710; df = 4, 44; $p < 0.001$) that of the uninfested control. *B. tabaci* adult infestation caused a slight increase in SA level, it was 1.38-fold (F = 31.887; df = 4, 44; $p < 0.001$) that of the uninfested control at densities of A4 (9–10 nymphs/cm^2), respectively (Table 1).

Table 1. Effects of infestation by whitefly adults and nymphs on defense phytohormone contents (ng/g FW) of systemic leaves of tobacco plants.

Phytohormone	Feeding Stage	*B. tabaci* Infestation Level				
		Control	N1/A1	N2/A2	N3/A3	N4/A4
Salicylic acid	Nymph	55.44 ± 0.84 ^{d}	61.17 ± 2.99 ^{d}	118.26 ± 4.11 ^{c}	145.73 ± 5.25 ^{a}	75.48 ± 1.39 ^{b}
	Adult	56.51 ± 1.19 ^{c}	57.62 ± 0.87 ^{c}	61.74 ± 1.33 ^{c}	69.91 ± 1.52 ^{a}	77.80 ± 2.54 ^{b}
Jasmonic acid	Nymph	36.05 ± 0.92 ^{a}	35.22 ± 0.99 ^{a,b}	29.82 ± 0.70 ^{c,d}	26.83 ± 1.32 ^{d}	31.91 ± 0.99 ^{b,c}
	Adult	35.11 ± 1.26 ^{b}	33.75 ± 1.10 ^{b}	35.93 ± 0.98 ^{a,b}	37.84 ± 1.38 ^{a,b}	41.81 ± 2.51 ^{a}

Each value represents the average (±SE) of three replicates. The different letters at the line after each datum indicate significant differences in phytohormone contents among plants treated for different densities ($p < 0.05$).

Feeding stage (F = 35.612; df = 1, 89; $p = 0.002$) and infestation density (F = 4.130; df = 4, 89; $p = 0.004$) had significant effects on JA levels in tobacco plants. *B. tabaci* nymph infestation significantly suppressed JA level, it was 0.74-fold (F =14.403; df = 4, 44; $p < 0.01$) that of control at densities of N3 (9–10 nymphs/cm^2). However, *B. tabaci* adult infestation cause a slight increase in JA level, it was 1.19-fold (F = 4.081; df = 4, 44; $p < 0.05$) that of the uninfested control at densities of A4 (400 ± 20/leaf) (Table 1).

3.4. Infestation of Whitefly Nymphs and Adults at Different Densities on the Expression of SA- and JA-Related Genes

B. tabaci nymph infestation significantly increased *PR-1a* and *PR-2a* expression, it was 3.02- (F = 4.710; df = 4, 14; $p < 0.05$) and 6.29-fold (F = 7.787; df = 4, 14; $p < 0.001$) that of control at densities of N3 (9–10 nymphs/cm^2), respectively. However, the expression of *PI-II* and *TPI* was obviously inhibited at the same feeding density, it was 0.393- (F = 5.222; df = 4, 14; $p < 0.05$) and 0.475-fold (F = 5.685; df = 4, 14; $p < 0.05$) that of control, respectively (Figure 3a). Feeding of *B. tabaci* adults caused a slight increase in *PR-1a* and *PR-2a* expression, which was 2.03- (F = 3.428; df = 4, 14; $p = 0.073$) and 1.77- (F = 1.911; df = 4, 14; $p = 0.235$) fold that of control at densities of A3 (200 ± 10/leaf), respectively. *B. tabaci* adult infestation had no significant effect on *PI-II* expression, except at the A4 (400 ± 20/leaf) density, which was 1.47-fold (F = 4.371; df = 4, 14; $p < 0.05$) that of control. No significant effect on *TPI* expression was detected at any of the four different feeding densities of *B. tabaci* adults (Figure 3b).

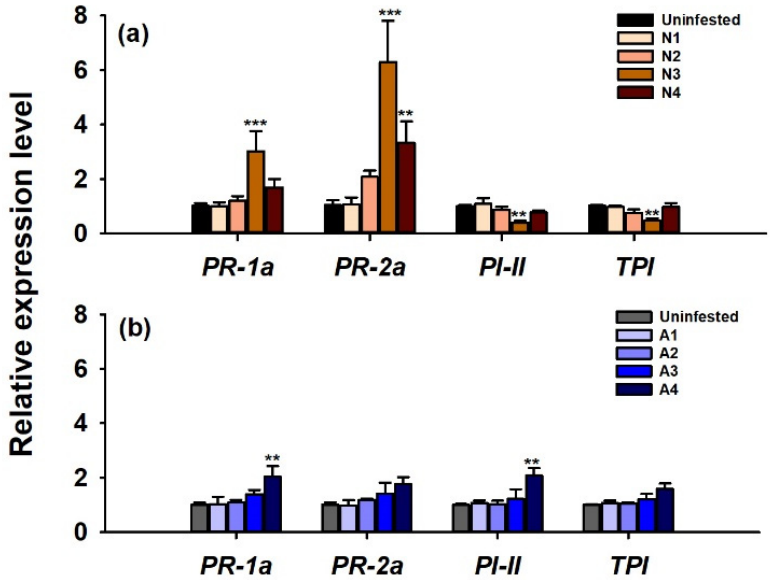

Figure 3. Relative expression of SA and JA pathway defense genes on systemic leaves of tobacco plants infested with *B. tabaci* MEAM1 nymphs (**a**) and adults (**b**). Asterisks indicate significant differences ($p \leq 0.05$). Error bars represent standard error of the means. The asterisks above the bars indicate significant differences (** $p < 0.05$, *** $p < 0.001$).

3.5. The Relationship between Saliva Effector Bt56 and Aphids Performance

Bt56 expression levels varied with the feeding stages of *B. tabaci*. A 2.14-fold (t = 3.524; df = 4; $p < 0.05$) higher expression level was detected in nymphs than in adults (Figure 4a).

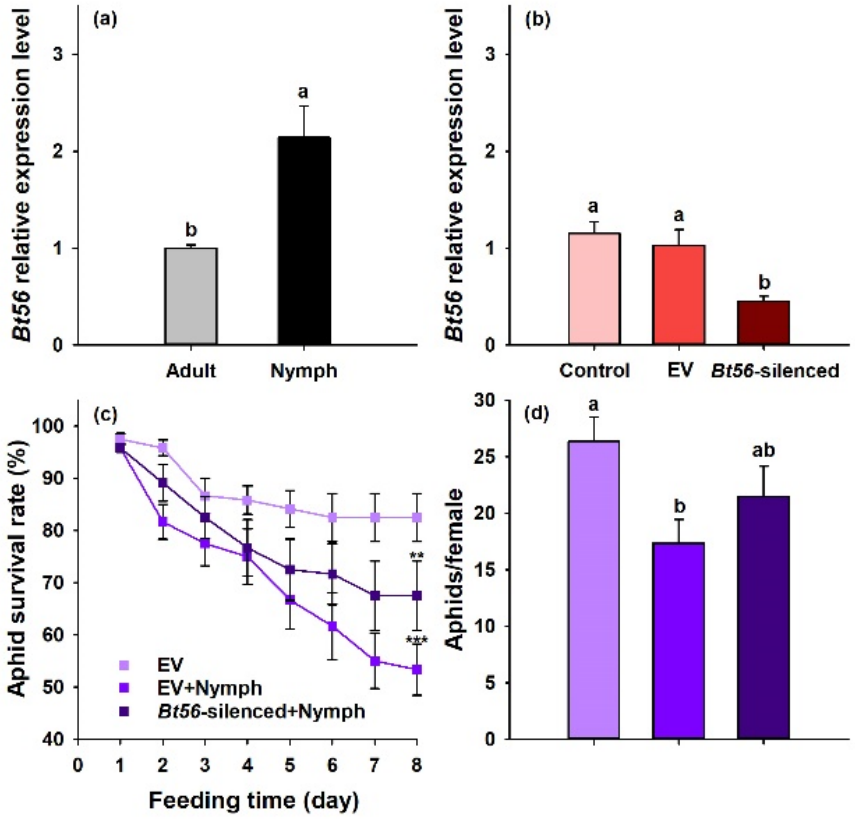

Figure 4. Effect of *Bt56* silencing on *M. persicae* performance. The transcript levels of *Bt56* in *B. tabaci* MEAM1 (**a**). The transcript levels of *Bt56* in *B. tabaci* nymphs feeding on pTRV2-*Bt56* tobacco (**b**). *M. persicae* survival (**c**) and fecundity (**d**) on *B. tabaci* MEAM1 nymph-infested tobacco plants. Error bars represent standard error of the means. The asterisks above the bars indicate significant differences (** $p < 0.05$, *** $p < 0.001$). The different letters above the bars indicate values that are significantly different ($p < 0.05$).

Bt56 of *B. tabaci* nymphs was silenced using the virus-induced gene silencing technique. Relative to expression in nymphs feeding on empty vector control tobacco plants, *Bt56* expression in nymphs feeding on pBIN2mDNA1-*Bt56* tobacco plants was reduced by 58.9% (F = 9.962; df = 2, 8; $p < 0.05$) (Figure 4b).

Bt56 silencing significantly affected the aphid resistance induced by *B. tabaci* nymphs. *M. persicae* survival was 35.3% (F = 7.069; df = 2, 17; $p < 0.05$) lower than the uninfested control on leaves of *B. tabaci* nymph-infested empty-vector-injected control plants, while only 18.1% (F = 7.069; df = 2, 17; $p = 0.164$) lower than uninfested control on *Bt56*-silenced plants (Figure 4c). Similarly, fecundity of *M. persicae* was 35.4% (F = 3.710; df = 2, 17; $p < 0.05$) and 17.5% (F = 3.710; df = 2, 17; $p = 0.336$) lower than the uninfested control on *B. tabaci* nymph-infested empty-vector-injected control and *Bt56*-silenced plants, respectively (Figure 4d).

4. Discussion

Plants quickly perceive various stress signals and then initiate signaling cascades and eventually activate the defense responses [36]. Difference in feeding type, duration, and even infestation level of insect herbivores are frequently cited reasons for different plant reactions [17,37]. In our performance assay, *B. tabaci* induced detrimental effects on *M. persicae* on tobacco that differed with the developmental stage. Pre-infestation of *B. tabaci*

MEAM1 nymphs significantly decreased the survival and fecundity of the subsequent *M. persicae*, whereas adult infestation had no significant effects on aphid performance. These results are consistent with those of previous studies showing that nymphs of *B. tabaci* biotype A induced higher levels of expression of several PR protein genes than the adult stage in tomato plants [26]. Additionally, *B. tabaci* MEAM1 nymphs, but not adults, induce accumulation of *SLW1* and *SLW3* RNAs [11] and cause squash leaf silvering disorder [13].

Plants deploy multiple defense-signaling pathways to antagonize insect herbivores [38,39]. To gain deep insight into the differences between nymph and adult whiteflies, whether the two feeding stages differ in induced defenses was tested, focusing mainly on the SA and JA pathways. In this study, *B. tabaci* nymph infestation significantly increased SA accumulation and transcript levels of SA-responsive genes, *PR-1a* and *PR-2a*, but suppressed JA synthesis and JA-responsive genes expression. In contrast, adult whiteflies infestation had much less effect on SA-related responses, and only slight increase in SA and related defense genes transcript level was observed, whereas small increase in JA signaling was detected after feeding of adult whiteflies. In line with our findings, in *Arabidopsis*, SA signal pathway is obviously activated after *B. tabaci* MEAM1 nymph infestation, while the JA signal pathway is either suppressed or not affected [25,40,41]. Zhang et al. (2018) found that tomato plants infested with *B. tabaci* MEAM1 adults had higher SA and JA levels than those of the control [42]. Furthermore, our performance results found that the introduction of *NahG* abolished the aphid resistance mediated by nymph whiteflies. The SA signal pathway has been involved in the defense of wheat in response to the Russian wheat aphid and that of tomato to *Macrosiphum euphorbiae* [43,44]. Similarly, in our previous studies, a positive correlation was found between *B. tabaci* MEAM1-induced aphid resistance and the SA-mediated defense [32,45]. Consequently, in the present study, feeding of adults and nymphs had widely divergent influences on the host defense responses, which could explain the discrepancies in aphid resistance.

The saliva of phloem feeders is a mediator of plant–insect interactions [29,46], and herbivores with different salivary components can evoke different plant responses [47,48]. According to Van de Ven et al. (2000), tomato or pumpkin silver leaf disorder was caused by specific salivary components of *B. tabaci* MEAM1 nymphs [11]. Puthoff et al. (2010) also indicated that the quality and quantity of saliva effectors in adults and nymphs may be different [30,49]. The important role of *B. tabaci* MED salivary effector *Bt56* in eliciting SA-related plant defenses has been proved [28,29]. In our qPCR assay, dramatically higher transcript level of *Bt56* was detected in *B. tabaci* nymphs than adults, which indicated a stronger stimulation induced by nymph vs. adult. Further research with virus-induced gene silencing technique proved that differences in *B. tabaci* salivary lead to differences in aphid resistance, as *Bt56* silencing partly impaired the aphid resistance induced by *B. tabaci* nymphs. The successful feeding of *B. tabaci* is likely enabled by combination of various saliva effectors. Up to now, more and more whitefly salivary proteins have been identified in recent years, such as LAC1, BtFer1, Bsp9, and BtArmet [28,29,50–52]. BtFer1 has been shown to enhance whitefly feeding by repressing jasmonic JA-mediated defense responses [28]. BtArmet acts to promote whitefly performance through binding to the proteinase inhibitors NtCYS6 on tobacco plants [52]. In particular, Bsp9, a virus-induced whitefly salivary effector, can effectively inhibit the plant defense response to benefit both whitefly fitness and virus spread [51]. All these salivary proteins may be involved in the complicated interaction among *B. tabaci*, host plants, and other phytophagous competitors.

Changes in herbivore density can also dictate the intensity of species interactions and may play a critical role in determining the outcome of plant-mediated herbivore interactions [16,53,54]. In this study, we found that *B. tabaci* MEAM1 nymph-induced aphid resistance was density-dependent. Aphid resistance was first detected at low–medium density and peaked at medium density, whereas neither the lowest nor the highest feeding densities of *B. tabaci* MEAM1 nymphs triggered significant aphid resistance. In addition to performance distinctions, biochemical and qPCR results indicated that the magnitude of the response mediated by nymph whiteflies varied with the infestation levels. The

strongest defense response in SA signaling was observed at medium density during nymph whiteflies infestation. Collectively, these results proved that medium density was the optimal density in triggering aphid resistance in tobacco. Messina et al. (2002) investigated the effect of different densities of pre-infested Russian wheat aphid on subsequent aphids and found that the negative influence was most obvious at medium density, with lower resistance detected at both low–medium and high densities, consistent with our results [55].

5. Conclusions

Induced plant defenses are complex and are affected by multiple factors. Based on this study, we conclude that infestation of *B. tabaci* MEAM1 nymphs and adults induced a difference in resistance to aphids in tobacco plants. The nymphs, but not the adults, significantly inhibited the survival and fecundity of subsequent *M. persicae* by triggering SA-related defenses. Furthermore, a density threshold was reached for this induced resistance. Both *B. tabaci* and *M. persicae* are phloem feeders and highly efficient virus vectors in nature. The very importance of salivary effectors for sap-sucking pest in manipulating host cell processes and promoting insect infestation and virus spread has been well established. Identifying specific novel salivary effectors in nymph whiteflies is a valuable contribution that will help not only to understand the replace mechanism of *B. tabaci* on *M. persicae* but also promote the development of novel strategies for pest management.

Author Contributions: H.Z. conceived the idea. Y.L. and X.Y. conducted the experiment. Z.Y. and C.Q. collected the literature review. Y.L., X.S. and C.Q. provided technical expertise to strengthen the basic idea. Y.L. and C.Q. helped in statistical analysis. H.Z. and Y.L. proofread and provided intellectual guidance. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the National Natural Science Foundation of China (31701799) and Shandong Modern Agricultural Industry Technology System (SDAIT05-022-08).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in this study are available on fair request to the corresponding author.

Acknowledgments: The authors are grateful to the Department of Plant Pathology, Nanjing Agricultural University, for providing tobacco seeds and gene silencing vectors and all the members of the Plant Disease Prevention Laboratory for technical assistance. The authors also thank the reviewers for their constructive comments and suggestions.

Conflicts of Interest: All other authors declare no conflict of interest.

References

1. Li, X.C.; Ni, X.Z. Deciphering the Plant-Insect Phenotypic Arms Race. *Recent Adv. Entomol. Res.* **2011**, 3–33. [CrossRef]
2. Kessler, A.; Baldwin, I.T. Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature. *Science* **2001**, *291*, 2141–2144. [CrossRef]
3. Lawrence, S.D.; Novak, N.G. Expression of Poplar Chitinase in Tomato Leads to Inhibition of Development in Colorado Potato Beetle. *Biotechnol. Lett.* **2006**, *28*, 593–599. [CrossRef]
4. Stam, J.M.; Kroes, A.; Li, Y.; Gols, R.; van Loon, J.J.; Poelman, E.H.; Dicke, M. Plant Interactions with Multiple Insect Herbivores: From Community to Genes. *Annu. Rev. Plant Biol.* **2014**, *65*, 689–713. [CrossRef]
5. Caarls, L.; Pieterse, C.; Van Wees, S.C.M. How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front. Plant Sci.* **2015**, *6*, 170. [CrossRef]
6. Lou, Y.G.; Cheng, J.A. Induced plant resistance to phytophagous insects. *Acta Entomol. Sin.* **1997**, *40*, 320–331.
7. Hebert, S.L.; Jia, L.; Goggin, F.L. Quantitative differences in aphid virulence and foliar symptom development on tomato plants carrying the Mi resistance gene. *Environ. Entomol.* **2007**, *36*, 458–467. [CrossRef]
8. Bostock, R.M. Signal Crosstalk and Induced Resistance: Straddling the Line Between Cost and Benefit. *Annu. Rev. Phytopathol.* **2005**, *43*, 545–580. [CrossRef]
9. Heil, M.; Ton, J. Long-distance signalling in plant defence. *Trends Plant Sci.* **2008**, *13*, 264–272. [CrossRef]
10. Kessler, A.; Baldwin, I.T. Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. *Plant J.* **2004**, *38*, 639–649. [CrossRef]

11. Van De Ven, W.T.G.; Levesque, C.S.; Perring, T.M.; Walling, L.L. Local and Systemic Changes in Squash Gene Expression in Response to Silverleaf Whitefly Feeding. *Plant Cell* **2000**, *12*, 1409. [CrossRef] [PubMed]
12. Kant, M.R.; Sabelis, M.W.; Haring, M.A.; Schuurink, R.C. Intraspecific variation in a generalist herbivore accounts for differential induction and impact of host plant defences. *Proc. R. Soc. B Boil. Sci.* **2007**, *275*, 443–452. [CrossRef] [PubMed]
13. Costa, H.S.; Brown, J.K.; Sivasupramaniam, S.; Bird, J. Regional distribution, insecticide resistance, and reciprocal crosses between the A and B biotypes of *Bemisia tabaci*. *Int. J. Trop. Insect Sci.* **1993**, *14*, 255–266. [CrossRef]
14. Thaler, J.S.; Stout, M.J.; Karban, R.; Duffey, S.S. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* **1996**, *22*, 1767–1781. [CrossRef] [PubMed]
15. Shiojiri, K.; Ozawa, R.; Kugimiya, S.; Uefune, M.; Van Wijk, M.; Sabelis, M.W.; Takabayashi, J. Herbivore-Specific, Density-Dependent Induction of Plant Volatiles: Honest or "Cry Wolf" Signals? *PLoS ONE* **2010**, *5*, e12161. [CrossRef] [PubMed]
16. Herrando-Pérez, S.; Delean, S.; Brook, B.W.; Bradshaw, C.J.A. Density dependence: An ecological Tower of Babel. *Oecologia* **2012**, *170*, 585–603. [CrossRef] [PubMed]
17. Hodge, S.; Bennett, M.; Mansfield, J.W.; Powell, G. Aphid-induction of defence-related metabolites in *Arabidopsis thaliana* is dependent upon density, aphid species and duration of infestation. *Arthropod-Plant Interact.* **2018**, *13*, 387–399. [CrossRef]
18. Kroes, A.; Broekgaarden, C.; Uribe, M.C.; May, S.; van Loon, J.J.A.; Dicke, M. Brevicoryne brassicae aphids interfere with transcriptome responses of *Arabidopsis thaliana* to feeding by *Plutella xylostella* caterpillars in a density-dependent manner. *Oecologia* **2016**, *183*, 107–120. [CrossRef]
19. Ponzio, C.; Gols, R.; Weldegergis, B.T.; Dicke, M. Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore. *Plant Cell Environ.* **2014**, *37*, 1924–1935. [CrossRef]
20. Kaplan, I.; Denno, R.F. Interspecific interactions in phytophagous insects revisited: A quantitative assessment of competition theory. *Ecol. Lett.* **2007**, *10*, 977–994. [CrossRef]
21. Liu, T.-X.; Oetting, R.D.; Buntin, G.D. Evidence of Interspecific Competition Between *Trialeurodes vaporariorum* (Westwood) and *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) on Some Greenhouse-Grown Plants. *J. Èntomol. Sci.* **1994**, *29*, 55–65. [CrossRef]
22. Pascual, S.; Callejas, C. Intra- and interspecific competition between biotypes B and Q of *Bemisia tabaci* (Hemiptera: Aleyrodidae) from Spain. *Bull. Èntomol. Res.* **2004**, *94*, 369–375. [CrossRef] [PubMed]
23. Inbar, M.; Doostdar, H.; Mayer, R.T. Effects of Sessile Whitefly Nymphs (Homoptera: Aleyrodidae) on Leaf-Chewing Larvae (Lepidoptera: Noctuidae). *Environ. Èntomol.* **1999**, *28*, 353–357. [CrossRef]
24. Luan, J.-B.; Li, J.-M.; Varela, N.; Wang, Y.-L.; Li, F.-F.; Bao, Y.-Y.; Zhang, C.-X.; Liu, S.-S.; Wang, X.-W. Global Analysis of the Transcriptional Response of Whitefly to *Tomato Yellow Leaf Curl China Virus* Reveals the Relationship of Coevolved Adaptations. *J. Virol.* **2011**, *85*, 3330–3340. [CrossRef] [PubMed]
25. Zhang, P.; Broekgaarden, C.; Zheng, S.; Snoeren, T.A.L.; Loon, J.J.A.; Gols, R.; Dicke, M. Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. *New Phytol.* **2013**, *197*, 1291–1299. [CrossRef] [PubMed]
26. Estrada-Hernández, M.G.; Valenzuela-Soto, J.H.; Ibarra-Laclette, E.; Délano-Frier, J.P. Differential gene expression in whitefly *Bemisia tabaci*-infested tomato (*Solanum lycopersicum*) plants at progressing developmental stages of the insect's life cycle. *Physiol. Plant.* **2009**, *137*, 44–60. [CrossRef]
27. Zhang, P.-J.; Zheng, S.-J.; van Loon, J.J.A.; Boland, W.; David, A.; Mumm, R.; Dicke, M. Whiteflies interfere with indirect plant defense against spider mites in Lima bean. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 21202–21207. [CrossRef]
28. Su, Q.; Peng, Z.; Tong, H.; Xie, W.; Wang, S.; Wu, Q.; Zhang, J.; Li, C.; Zhang, Y. A salivary ferritin in the whitefly suppresses plant defenses and facilitates host exploitation. *J. Exp. Bot.* **2019**, *70*, 3343–3355. [CrossRef]
29. Xu, H.-X.; Qian, L.-X.; Wang, X.-W.; Shao, R.-X.; Hong, Y.; Liu, S.-S.; Wang, X.-W. A salivary effector enables whitefly to feed on host plants by eliciting salicylic acid-signaling pathway. *Proc. Natl. Acad. Sci. USA* **2018**, *116*, 490–495. [CrossRef]
30. Yan, Y.; Peng, L.; Liu, W.-X.; Wan, F.-H.; Harris, M.K. Host Plant Effects on Alkaline Phosphatase Activity in the Whiteflies, *Bemisia tabaci* Biotype B and *Trialeurodes vaporariorum*. *J. Insect Sci.* **2011**, *11*, 9. [CrossRef]
31. Xue, M.; Wang, C.-X.; Bi, M.-J.; Li, Q.-L.; Liu, T.-X. Induced Defense by *Bemisia tabaci* Biotype B (Hemiptera: Aleyrodidae) in Tobacco against *Myzus persicae* (Hemiptera: Aphididae). *Environ. Èntomol.* **2010**, *39*, 883–891. [CrossRef] [PubMed]
32. Zhang, X.; Xue, M.; Zhao, H. Species-specific effects on salicylic acid content and subsequent Myzus persicae (Sulzer) performance by three phloem-sucking insects infesting *Nicotiana tabacum* L. *Arthropod-Plant Interact.* **2015**, *9*, 383–391. [CrossRef]
33. Ishaaya, I.; De Cock, A.; Degheele, D. Pyriproxyfen, a Potent Suppressor of Egg Hatch and Adult Formation of the Greenhouse Whitefly (Homoptera: Aleyrodidae). *J. Econ. Èntomol.* **1994**, *87*, 1185–1189. [CrossRef]
34. Alon, M.; Malka, O.; Eakteiman, G.; Elbaz, M.; Ben Zvi, M.M.; Vainstein, A.; Morin, S. Activation of the Phenylpropanoid Pathway in Nicotiana tabacum Improves the Performance of the Whitefly *Bemisia tabaci* via Reduced Jasmonate Signaling. *PLoS ONE* **2013**, *8*, e76619. [CrossRef]
35. You-Ming, Y.; Chu-Nian, X.; Bao-Min, W.; Jun-Zhen, J. Effects of plant growth regulators on secondary wall thickening of cotton fibres. *Plant Growth Regul.* **2001**, *35*, 233–237. [CrossRef]
36. Wang, X.-W.; Li, P.; Liu, S.-S. Whitefly interactions with plants. *Curr. Opin. Insect Sci.* **2017**, *19*, 70–75. [CrossRef]
37. Pinto-Zevallos, D.M.; Bezerra, R.H.S.; Souza, S.R.; Ambrogi, B.G. Species- and density-dependent induction of volatile organic compounds by three mite species in cassava and their role in the attraction of a natural enemy. *Exp. Appl. Acarol.* **2018**, *74*, 261–274. [CrossRef]

38. Glazebrook, J. Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annu. Rev. Phytopathol.* **2005**, *43*, 205–227. [CrossRef]
39. Walling, L.L. Chapter 13 Adaptive Defense Responses to Pathogens and Insects. *Plant Innate Immun.* **2009**, *51*, 551–612. [CrossRef]
40. Zarate, S.I.; Kempema, L.A.; Walling, L.L. Silverleaf Whitefly Induces Salicylic Acid Defenses and Suppresses Effectual Jasmonic Acid Defenses. *Plant Physiol.* **2006**, *143*, 866–875. [CrossRef]
41. Kempema, L.A.; Cui, X.; Holzer, F.M.; Walling, L.L. Arabidopsis Transcriptome Changes in Response to Phloem-Feeding Silverleaf Whitefly Nymphs. Similarities and Distinctions in Responses to Aphids. *Plant Physiol.* **2006**, *143*, 849–865. [CrossRef] [PubMed]
42. Zhang, P.-J.; He, Y.-C.; Zhao, C.; Ye, Z.-H.; Yu, X.-P. Jasmonic Acid-Dependent Defenses Play a Key Role in Defending Tomato Against *Bemisia tabaci* Nymphs, but Not Adults. *Front. Plant Sci.* **2018**, *9*, 1065. [CrossRef] [PubMed]
43. Mohase, L.; van der Westhuizen, A.J. Salicylic acid is involved in resistance responses in the Russian wheat aphid-wheat interaction. *J. Plant Physiol.* **2002**, *159*, 585–590. [CrossRef]
44. Avila, C.A.; Arévalo-Soliz, L.M.; Jia, L.; Navarre, D.A.; Chen, Z.; Howe, G.A.; Meng, Q.-W.; Smith, J.E.; Goggin, F.L. Loss of Function of FATTY ACID DESATURASE7 in Tomato Enhances Basal Aphid Resistance in a Salicylate-Dependent Manner. *Plant Physiol.* **2012**, *158*, 2028–2041. [CrossRef] [PubMed]
45. Zhao, H.; Zhang, X.; Xue, M.; Zhang, X. Feeding of Whitefly on Tobacco Decreases Aphid Performance via Increased Salicylate Signaling. *PLoS ONE* **2015**, *10*, e0138584. [CrossRef] [PubMed]
46. Blanc, S.; Uzest, M.; Drucker, M. New research horizons in vector-transmission of plant viruses. *Curr. Opin. Microbiol.* **2011**, *14*, 483–491. [CrossRef] [PubMed]
47. Tjallingii, W.F. Salivary secretions by aphids interacting with proteins of phloem wound responses. *J. Exp. Bot.* **2006**, *57*, 739–745. [CrossRef]
48. Yan, Y.; Zhang, H.; Yang, Y.; Zhang, Y.; Guo, J.; Liu, W.; Wan, F. Plant defense responses induced by *Bemisia tabaci* Middle East-Asia Minor 1 salivary components. *Èntomol. Exp. Appl.* **2016**, *159*, 287–297. [CrossRef]
49. Puthoff, D.P.; Holzer, F.M.; Perring, T.M.; Walling, L.L. Tomato Pathogenesis-related Protein Genes are Expressed in Response to *Trialeurodes vaporariorum* and *Bemisia tabaci* Biotype B Feeding. *J. Chem. Ecol.* **2010**, *36*, 1271–1285. [CrossRef]
50. Yang, C.-H.; Guo, J.-Y.; Chu, D.; Ding, T.-B.; Wei, K.-K.; Cheng, D.-F.; Wan, F.-H. Secretory laccase 1 in *Bemisia tabaci* MED is involved in whitefly-plant interaction. *Sci. Rep.* **2017**, *7*, 3623. [CrossRef]
51. Wang, N.; Zhao, P.; Ma, Y.; Yao, X.; Sun, Y.; Huang, X.; Jin, J.; Zhang, Y.; Zhu, C.; Fang, R.; et al. A whitefly effector Bsp9 targets host immunity regulator WRKY33 to promote performance. *Philos. Trans. R. Soc. B Biol. Sci.* **2019**, *374*, 20180313. [CrossRef] [PubMed]
52. Du, H.; Xu, H.; Wang, F.; Qian, L.; Liu, S.; Wang, X. Armet from whitefly saliva acts as an effector to suppress plant defenses by targeting tobacco cystatin. *New Phytol.* **2022**. [CrossRef] [PubMed]
53. Underwood, N. Density dependence in insect performance within individual plants: Induced resistance to Spodoptera exigua in tomato. *Oikos* **2010**, *119*, 1993–1999. [CrossRef]
54. Kroes, A.; Van Loon, J.J.; Dicke, M. Density-Dependent Interference of Aphids with Caterpillar-Induced Defenses in Arabidopsis: Involvement of Phytohormones and Transcription Factors. *Plant Cell Physiol.* **2014**, *56*, 98–106. [CrossRef] [PubMed]
55. Messina, F.J.; Taylor, R.; Karren, M.E. Divergent responses of two cereal aphids to previous infestation of their host plant. *Èntomol. Exp. Appl.* **2002**, *103*, 43–50. [CrossRef]