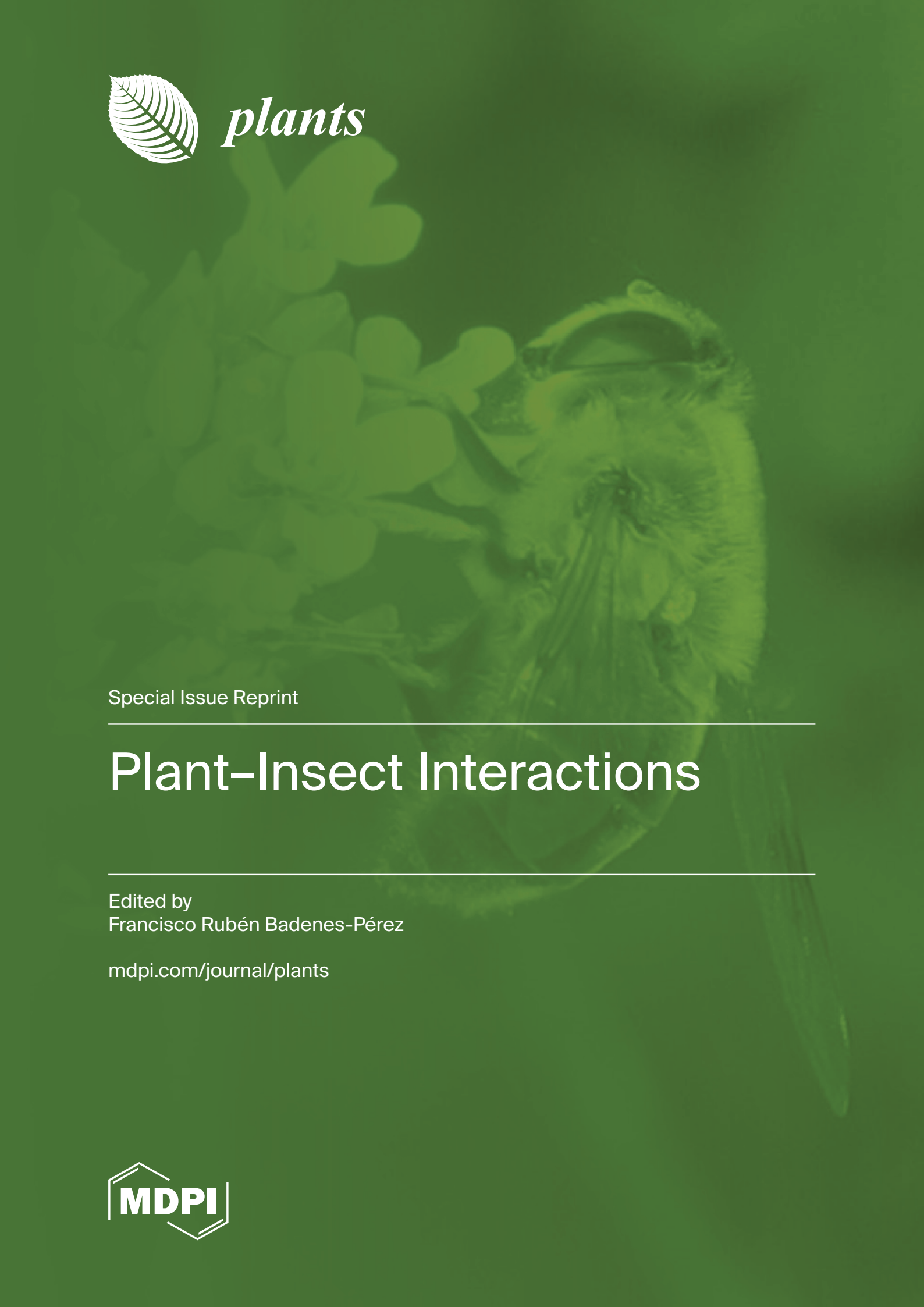

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Special Issue Reprint

Plant–Insect Interactions

Edited by
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Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

This is a reprint of the Special Issue, published open access by the journal *Plants* (ISSN 2223-7747), freely accessible at: https://www.mdpi.com/journal/plants/special_issues/plant_insect.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-5273-4 (Hbk)
ISBN 978-3-7258-5274-1 (PDF)
https://doi.org/10.3390/books978-3-7258-5274-1

Cover image courtesy of Francisco Rubén Badenes-Pérez

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Editorial

Plant-Insect Interactions

Francisco Rubén Badenes-Pérez

Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain; fr.badenes@csic.es

1. Introduction

The central part of the study of plant-insect interactions comes from our quest for knowledge on why and how these interactions occur. Within the broad topic of plant-insect interactions, insect pest management and insect plant pollination are among the most relevant research areas because of the economic impact that they can have on crop yield [1–5]. This Special Issue presents a collection of papers dealing with basic and applied topics in plant-insect interactions, but showing the relative importance that pest management and pollination have in this field.

2. Key Messages

Host-plant resistance is a prominent part of integrated pest management [6]. In Brassicae, glucosinolates play a key role in host-plant resistance [7]. Three papers in this Special Issue deal with glucosinolates and host-plant resistance to insect pests [8–10]. Genotypes of kale *Brassica oleracea* L. var. *acephala* (Brassicaceae) that have different glucosinolate content can differ in glucosinolate induction and resistance to the cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) [8]. Another generalist lepidopteran, the cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), can metabolize glucosinolates via conjugation to glutathione, however a high content of nitrogen and sulphur amino acid contents in the plant facilitates this process [9,11]. *Helicoverpa armigera*, however, does not seem to improve its performance on plants containing glucosinolates after selection for glucosinolate adaptation [10]. Despite this, *H. armigera* has a pest status in Brassicaceae, and this is also due to other factors, such as the damage that this insect causes to plant reproductive structures [12], insecticide resistance [13], and suppression of natural enemies by insecticide use [14].

In gladiolus *Gladiolus hybridus* L. (Iridaceae), host-plant resistance to western flower thrips *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) can be conferred by morphological factors, such as the density of epicuticular papillae, and by chemical factors, such as content of triterpenoid saponins [15]. Triterpenoid saponins have also been suggested as being involved in resistance to *F. occidentalis* in a different plant, *Barbarea vulgaris* R. Br. (Brassicaceae) [16].

Insect population dynamics are greatly affected by weather conditions [17–19]. Bažok et al. show that corn damage caused by the first generation of European corn borer *Ostrinica nubilalis* Hübner (Lepidoptera: Crambidae) is positively correlated with air temperature and negatively correlated with air humidity [20]. Fertilizer use can also affect insect preference and development on plants [21–23]. In this regard, Li et al. show that the white-backed planthopper *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae) prefers plants grown in substrate containing high content of nitrogen fertilizer, developing also faster and having a longer lifespan when feeding on plants grown under high nitrogen conditions [24]. Endophytic microorganisms can also influence plant-insect interactions and the plant response to herbivory [25,26]. *Bacillus subtilis* 26D Cohn secretes cytokinins that help plantlets of potato *Solanum tuberosum* L. (Solanaceae) recover after herbivory by the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) [27].

Pollinators and natural enemies of pests are essential for food production [28–32]. Because of this, insectary plants can be used for conservation biological control and pollinator conservation [33,34]. The paper by Kati et al. shows that some wildflowers planted in the field margins of tomato fields attract high numbers of wild bees, honeybees, and parasitoids [35]. Their research also shows that flower cover is correlated with the abundance of wild bees and honeybees. Tomato is one of the crops that requires insect pollination in order to maximize yield [36] and, therefore, the presence of wildflowers that attract pollinators is likely to have a positive effect on tomato yield. The paper by Kati et al. also includes some plant species that have not been previously tested as insectary plants. The study of insectary plants often focuses on few plant species that have often been proven to fulfill their purpose, such as buckwheat *Fagopyrum esculentum* Moench (Polygonaceae), *Lobularia maritima* L. Desv. (Brassicales), and *Phacelia tanacetifolia* Benth. (Boraginaceae) [37–39]. In terms of understanding the mechanisms of attraction of flowers to pollinators, Giuliani et al. show that glandular trichomes and monoterpene VOCs could be involved in attracting pollinators to two *Salvia* spp., *S. blepharophylla* Brandegee ex Epling and *S. greggii* A. Gray (Lamiaceae) [40].

3. Future Directions

Plant-insect interactions include a tremendous diversity of relationships deserving further research [41,42]. In these interactions, plant secondary metabolites can play an important role [43–45]. In this Special Issue, different chemical compounds were studied for their role in host-plant resistance. However, identification of additional chemical compounds involved in plant-insect interactions continues to be of importance. For example, in Brassicaceae, a lot of the research conducted in plant-insect interactions in the context of chemical ecology has been conducted with glucosinolates [7,46,47]. Future research is necessary to study the role that other plant secondary metabolites may play in plant-insect interactions that have so far been studied mostly by measuring glucosinolate content. The paper by Jeschke et al. [9] indicates that content of other plant compounds, such as amino acids, can affect the ability of the insect to detoxify plant secondary metabolites. Furthermore, the identification of the two unidentified triterpenoid saponins that seem to confer gladiolus resistance to *F. occidentalis* [15] could uncover additional compounds of importance in host-plant resistance to this and other herbivores. It should also be investigated if there are ontogenetic and seasonal changes in saponin content in gladiolus. In other plant species, based on the changes observed in insect resistance, ontogenetic, phenological, and seasonal changes in saponin content seem to occur [48–50]. Moreover, the effect of bacteria on plant repair mechanisms after insect herbivory and plant-insect interactions in general also deserves being subject to further research. In terms of pollination, after having tested the effect of wildflowers on attraction to pollinators and natural enemies, as in the case of the margins of tomato fields [35], further research should measure how the presence of wildflowers affects fruit quality and yield.

Acknowledgments: I thank the authors who contributed to this Special Issue.

Conflicts of Interest: The author declares no conflict of interest.

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Article

Glucosinolate Induction and Resistance to the Cabbage Moth, *Mamestra brassicae*, Differs among Kale Genotypes with High and Low Content of Sinigrin and Glucobrassicin

Francisco Rubén Badenes-Pérez [1,*] and María Elena Cartea [2]

[1] Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain
[2] Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas, 36080 Pontevedra, Spain; ecartea@mbg.csic.es
* Correspondence: fr.badenes@csic.es

Abstract: The cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae), is a generalist insect pest of cruciferous crops. We tested glucosinolate induction by jasmonic acid (JA) and salicylic acid (SA), and by these phytohormones combined with feeding by *M. brassicae* larvae in four genotypes of kale, *Brassica oleracea* L. var. *acephala* (Brassicaceae). The genotypes tested had high glucobrassicin (genotype HGBS), low glucobrassicin (genotype LGBS), high sinigrin (genotype HSIN), and low sinigrin content (genotype LSIN). Application of JA increased indolic and total glucosinolate content in all kale genotypes 1, 3, and 9 days after treatment. For SA-treated plants, glucosinolate induction varied depending on the number of days after treatment and the genotype. Overall, herbivory by *M. brassicae* accentuated and attenuated the effects of JA and SA, respectively, on plant glucosinolate content. Larvae of *M. brassicae* gained less weight on leaves from plants treated with JA compared to leaves from control plants and plants treated with SA. In bioassays with leaf discs, a significant reduction of defoliation only occurred in JA-treated plants of the HSIN genotype. This research shows that previous herbivory alters the susceptibility of kale to *M. brassicae* and that induction of glucosinolates varies among kale genotypes differing in their glucosinolate content.

Keywords: *Brassica oleracea* var. *acephala*; glucosinolates; herbivory; host-plant resistance; jasmonic acid; salicylic acid

1. Introduction

Plants in the family Brassicaceae contain glucosinolates that can be used for plant defense [1,2]. Unlike insects, specialists that are well adapted and even favored by glucosinolates in their host-plants, generalists are usually negatively affected by glucosinolate content [3–8]. This is the case for the cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae), for which high concentrations of glucosinolates have a detrimental effect on larval growth and survival [9–12]. For the development of larvae of this generalist, aliphatic glucosinolates have been shown to be more detrimental than indolic glucosinolates [13]. The aliphatic glucosinolates gluconapin, glucoiberin, and sinigrin have been associated to reduced performance of *M. brassicae* on different populations of *Brassica oleracea* L. (Brassicaceae) [14,15]. Experiments with different genotypes of kale, *B. oleracea* L. var. *acephala* (Brassicaceae), differing in the content of the aliphatic glucosinolates sinigrin and glucoiberin, and the indolic glucosinolate glucobrassicin, indicated that high content of these glucosinolates negatively affected larval weight in *M. brassicae* [11].

Jasmonic acid (JA) and salicylic acid (SA) modulate plant defense against different herbivores [16–18]. JA induces resistance against chewing herbivores, while phloem-feeders, which produce less injury to plant foliage, are perceived as pathogens and activate the SA signaling pathway [19]. Thus, JA and SA application can be used to simulate herbivory. Previous herbivory can affect plant responses to subsequent herbivory [20]. For

example, in *B. oleracea*, previous attack by the phloem-feeder aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) facilitated the herbivory in the chewing larvae of *Pieris rapae* L. (Lepidoptera: Pieridae), which developed faster and gained more weight on plants previous infested by *B. brassicae* [20].

JA and SA can affect induction of glucosinolates differently [21,22]. In *Brassica rapa* L. (Brassicaceae), SA application caused a greater increase in the aliphatic and aromatic glucosinolates sinigrin and gluconasturtiin, respectively, but a lesser increase in the indolic glucosinolates glucobrassicin and 4-methoxyglucobrassicin compared to JA application [23]. Feeding by lepidopteran larvae can induce glucosinolate content in plants [4,24,25]. In *Brassica napus* L. (Brassicaceae), feeding by *M. brassicae* larvae increased levels of the indolic glucosinolates glucobrassicin and neoglucobrassicin [26]. Thus, we expect that application of JA and SA will differently affect plant glucosinolate content and herbivory by *M. brassicae*. In the case of a chewing herbivore like *M. brassicae* larvae, JA application should have a more detrimental effect than SA application.

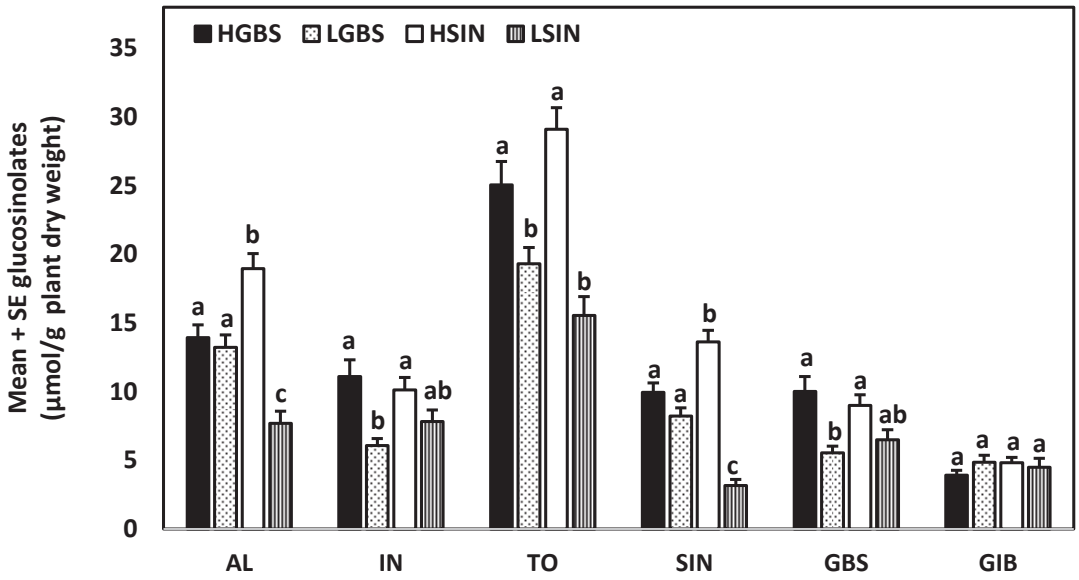
The objectives of this research were to compare glucosinolate induction, herbivory, and larval growth of *M. brassicae* after JA and SA application in four different plant genotypes selected for high and low glucobrassicin and sinigrin content. We also studied glucosinolate induction under the combination of either JA or SA application and feeding by *M. brassicae* larvae. In plants previously treated by these phytohormones, herbivory by *M. brassicae* is likely to boost and offset the effects of JA of SA, respectively. After treatment, we compared how glucosinolate changed through time 1, 3, and 9 days after treatment. We also compared the effect of each treatment on the glucosinolate content in each genotype at each time of analysis, and the differences in glucosinolate content among genotypes for each treatment and time of analysis. In the latter case, besides the actual glucosinolate content, we also looked at glucosinolate changes as a percentage of variation compared to the control within each genotype.

2. Results

2.1. Glucosinolate Content in Kale Genotypes

Analyzed over the length of the study, i.e., all control plants in each of the four kale genotypes combining the three time points, plants of the different genotypes differed in total aliphatic (AL), total indolic (IN), and total glucosinolate content (TO) ($p \leq 0.001$), as well as in sinigrin (SIN) ($p \leq 0.001$), glucobrassicin (GBS) ($p \leq 0.001$), neoglucobrassicin (NEO) ($p = 0.036$), and progoitrin (PRO) ($p \leq 0.001$) (Figure 1). Differences in the content of glucoiberin (GIB) ($p = 0.540$), 4-hydroxyglucobrassicin (OHGBS) ($p = 0.612$), 4-methoxyglucobrassicin (MEOHGBS) ($p = 0.075$), and gluconasturtiin (GNT) ($p = 0.212$) were not statistically significant. AL content was significantly higher in the HSIN genotype and significantly lower in LSIN than in the other genotypes. IN content was significantly higher in the HGBS and HSIN genotypes than in the LGBS genotype. TO content was higher in the genotypes HGBS and HSIN than in the genotypes LGBS and LSIN. Among individual glucosinolates, SIN content was higher in the genotype HSIN and lower in the genotype LSIN than in the other genotypes (Figure 1). This is the reason why we refer to these genotypes as HSIN and LSIN. GBS content was significantly higher in the HGBS and HSIN genotypes than in the LGBS genotype (Figure 1). Because of this, we refer to the other two genotypes used in this study as HGBS and LGBS. PRO content was higher in the genotype HSIN than in the genotypes HGBS and LSIN. In the experiments that we describe hereafter, we will focus on the main glucosinolate groups AL, IN, and TO. The most abundant glucosinolates GIB, SIN, GBS, and NEO are shown in the text only when comparisons of glucosinolate content among treatments and genotypes are shown as percentage increases (Table 1) and also in tables as supplementary data. The other less abundant glucosinolates are only shown as supplementary data (Table S1).

(**A**)



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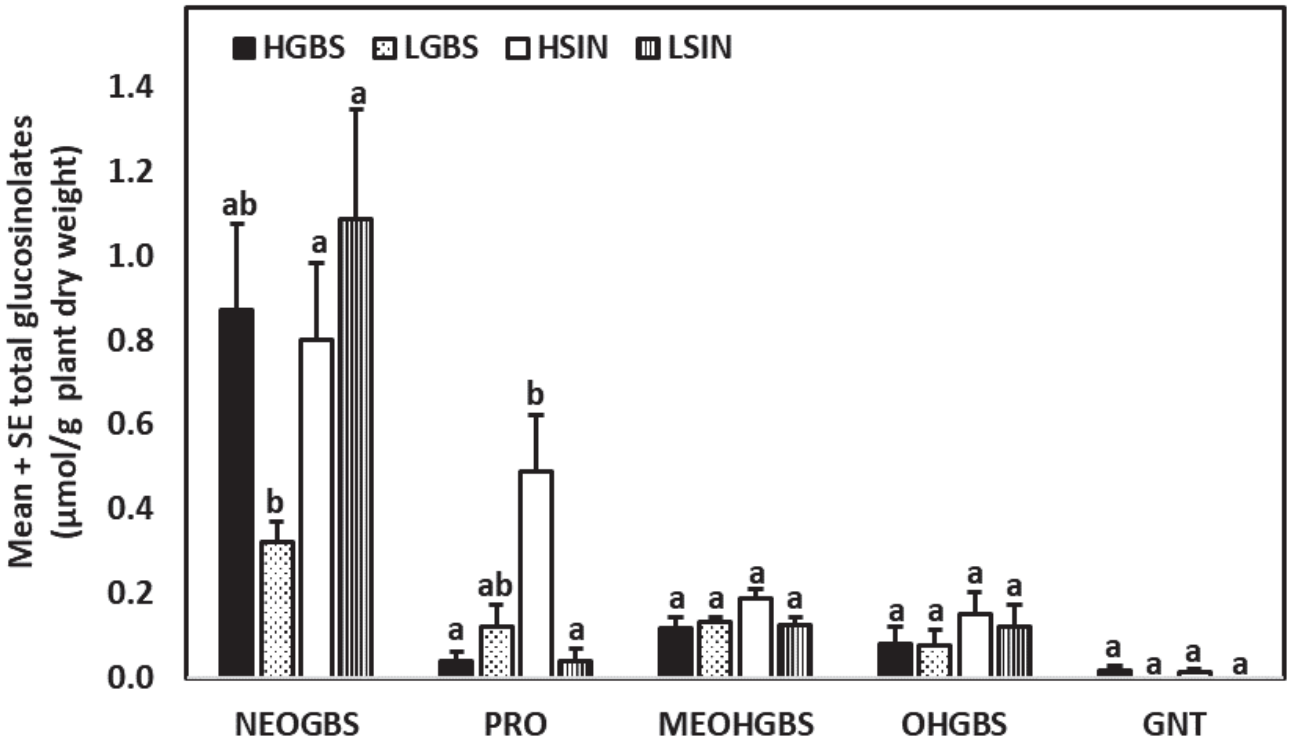


Figure 1. Mean ± SE glucosinolate content (µmol g^{-1} plant dry weight) in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). The glucosinolates shown are total aliphatic (AL), total indolic (IN), total glucosinolates (TO), sinigrin (SIN), glucobrassicin (GBS), and glucoiberin (GIB) (**A**), and neoglucobrassicin (NEO), progoitrin (PRO), 4-methoxyglucobrassicin (MEOHGBS), 4-hydroxyglucobrassicin (OHGBS), and gluconasturtiin (GNT) (**B**). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences in glucosinolate content among genotypes. Significant differences are shown with different lowercase letters.

Table 1. Mean ± SE percentage change in glucosinolate content (%) for each genotype and treatment one day after the application of the phytohormones (n = 3–10). The treatments are jasmonic acid (JA), salicylic acid (SA), JA with *Mamestra brassicae* larvae (JAL), SA with *M. brassicae* larvae (SAL), and control with *M. brassicae* larvae (CL). The genotypes are high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). The glucosinolates shown are glucoiberin (GIB), sinigrin (SIN), glucobrassicin (GBS), neoglucobrassicin (NEO), total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). For each time (days after treatment) and treatment, means within a column followed by different letters show significant differences ($p \leq 0.05$) among genotypes. Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively.

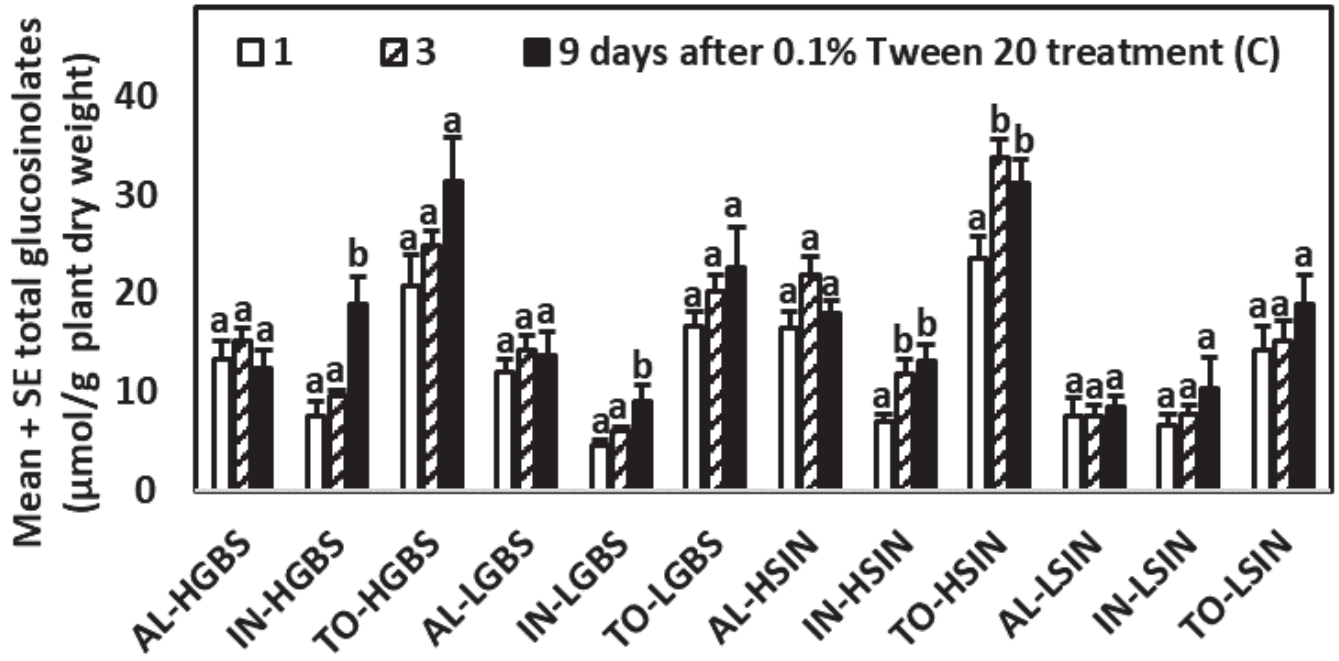
Days after Treat.	Tr.	Genotype	GIB	SIN	GBS	NEO	AL	IN	TO
1	JA	HGBS	69.3 ± 30.7 a	43.4 ± 17.5 a	195.7 ± 41.1 a	2198.0 ± 492.8 a	51.1 ± 17.7 a	292.3 ± 46.4 a	142.0 ± 21.7 a
		LGBS	60.8 ± 26.2 a	48.5 ± 29.8 a	320.9 ± 70.0 a	655.2 ± 139.4 b	50.8 ± 27.4 a	335.7 ± 64.5 a	132.7 ± 29.5 a
		HSIN	24.2 ± 18.6 a	−29.1 ± 10.1 a	171.8 ± 30.7 a	1584.8 ± 260.9 a	−17.2 ± 10.7 b	216.6 ± 29.5 ab	52.1 ± 13.3 b
		LSIN	−46.4 ± 6.9 b	37.4 ± 58.4 a	147.6 ± 36.6 a	230.6 ± 87.7 b	−12.6 ± 24.8 b	151.9 ± 32.1 b	65.0 ± 24.1 b
	SA	HGBS	97.2 ± 25.1 a	38.5 ± 17.8 a	29.3 ± 21.1 a	94.5 ± 46.1 ab	58.9 ± 11.3 a	37.7 ± 19.0 ab	51.3 ± 8.6 a
		LGBS	82.6 ± 21.7 a	77.1 ± 33.7 a	47.5 ± 20.3 a	171.8 ± 51.5 b	78.2 ± 23.6 a	51.6 ± 19.5 a	70.9 ± 19.7 a
		HSIN	10.0 ± 12.2 b	13.3 ± 16.7 a	−6.5 ± 11.8 a	5.7 ± 25.5 a	11.0 ± 12.1 b	−5.0 ± 34.4 b	6.2 ± 10.6 b
		LSIN	−18.4 ± 13.5 b	38.5 ± 28.3 a	5.5 ± 12.2 a	−9.7 ± 34.7 a	−0.7 ± 16.8 b	−0.3 ± 10.6 b	−0.5 ± 12.1 b
3	JA	HGBS	−32.3 ± 11.7 a	−43.7 ± 10.1 a	355.3 ± 40.1 a	2837.5 ± 777.7 a	−40.4 ± 9.8 a	514.4 ± 54.0 ac	176.2 ± 21.9 a
		LGBS	−26.6 ± 14.2 a	−19.1 ± 15.3 a	300.4 ± 50.5 a	498.6 ± 222.2 b	−20.1 ± 13.1 a	313.6 ± 49.4 b	79.7 ± 19.1 b
		HSIN	−42.8 ± 8.9 a	−49.7 ± 8.1 a	274.2 ± 55.4 a	1363.6 ± 292.9 ab	−48.6 ± 7.0 a	357.1 ± 46.6 ab	93.6 ± 18.2 b
		LSIN	−13.6 ± 14.6 a	−49.1 ± 13.6 a	376.1 ± 71.3 a	2145.0 ± 625.1 a	−27.9 ± 10.5 a	558.1 ± 75.1 c	269.9 ± 37.0 c
	JAL	HGBS	−3.1 ± 15.7 a	−54.2 ± 14.5 a	568.9 ± 86.5 a	3655.5 ± 709.6 a	−39.2 ± 14.6 a	763.2 ± 113.1 a	273.8 ± 50.0 a
		LGBS	−4.0 ± 18.3 a	−27.1 ± 18.7 a	545.6 ± 132.7 a	1039.6 ± 257.5 a	−18.0 ± 8.8 a	570.8 ± 124.4 a	158.1 ± 33.2 b
		HSIN	−37.3 ± 19.1 a	−37.1 ± 13.4 a	118.2 ± 62.5 b	2051.9 ± 669.5 a	−38.8 ± 9.0 a	265.3 ± 27.9 b	67.7 ± 8.4 c
		LSIN	−12.7 ± 24.0 a	−44.4 ± 13.1 a	201.3 ± 74.2 b	3106.3 ± 933.5 a	−13.7 ± 16.7 a	492.8 ± 71.3 ab	243.8 ± 32.1 ab
	SA	HGBS	−10.7 ± 12.4 a	16.8 ± 15.3 a	71.4 ± 26.5 a	64.0 ± 45.9 a	26.1 ± 27.4 a	83.9 ± 27.7 a	82.3 ± 56.1 a
		LGBS	10.9 ± 17.2 a	16.3 ± 14.7 a	55.2 ± 17.0 a	10.4 ± 19.6 a	15.2 ± 11.4 a	60.1 ± 15.8 a	28.6 ± 10.0 a
		HSIN	24.0 ± 14.3 a	10.5 ± 10.3 a	27.1 ± 10.3 a	−3.8 ± 30.4 a	12.5 ± 7.0 a	29.0 ± 11.3 a	18.2 ± 7.2 a
		LSIN	−8.9 ± 20.7 a	8.5 ± 15.8 a	50.4 ± 35.1 a	49.1 ± 36.7 a	2.1 ± 15.2 a	52.9 ± 35.9 a	28.7 ± 23.3 a
	SAL	HGBS	−3.9 ± 17.5 a	7.0 ± 12.2 a	129.1 ± 38.0 a	458.4 ± 75.9 a	3.8 ± 11.1 a	150.7 ± 36.1 a	60.9 ± 16.9 a
		LGBS	34.2 ± 10.4 a	33.2 ± 26.2 a	276.3 ± 76.6 a	662.8 ± 168.0 a	33.6 ± 17.0 a	300.2 ± 72.8 a	113.3 ± 31.5 a
		HSIN	22.0 ± 14.9 a	−16.2 ± 20.1 a	90.3 ± 26.0 a	1397.6 ± 642.7 a	−8.6 ± 16.2 a	190.9 ± 64.9 a	61.3 ± 14.9 a
		LSIN	2.7 ± 15.1 a	−14.9 ± 32.8 a	173.1 ± 84.3 a	1314.8 ± 718.6 a	−5.4 ± 17.3 a	286.9 ± 50.6 a	143.8 ± 26.1 a
	CL	HGBS	−9.7 ± 7.5 a	−53.8 ± 6.2 a	−11.6 ± 15.3 a	558.6 ± 164.9 a	−40.9 ± 3.8 a	25.9 ± 22.3 a	−12.9 ± 9.0 a
		LGBS	24.6 ± 6.6 a	−16.2 ± 10.2 a	130.0 ± 27.0 b	467.1 ± 73.6 a	−18.3 ± 7.0 a	151.3 ± 27.6 b	32.4 ± 7.9 b
		HSIN	−47.8 ± 14.5 a	−42.2 ± 17.9 a	113.4 ± 23.4 b	439.3 ± 224.6 a	−44.4 ± 16.0 a	137.3 ± 34.7 ab	20.6 ± 12.3 ab
		LSIN	−23.8 ± 13.5 a	−35.7 ± 10.9 a	268.7 ± 69.1 c	740.9 ± 216.6 a	−28.9 ± 6.6 a	314.6 ± 71.6 c	146.1 ± 39.2 c
9	JA	HGBS	55.1 ± 34.2 a	−28.9 ± 21.1 a	21.5 ± 13.8 a	177.1 ± 51.3 a	−7.0 ± 14.4 a	39.6 ± 12.2 a	21.2 ± 11.9 a
		LGBS	63.9 ± 45.7 a	−6.7 ± 19.6 a	42.6 ± 30.9 a	746.9 ± 256.8 b	14.2 ± 20.0 a	65.7 ± 25.5 a	34.7 ± 18.8 ab
		HSIN	25.1 ± 14.9 a	45.4 ± 16.7 a	94.8 ± 23.0 a	252.1 ± 103.1 a	37.1 ± 11.1 a	118.5 ± 20.9 a	71.4 ± 15.0 bc
		LSIN	28.0 ± 35.2 a	10.8 ± 32.9 a	142.8 ± 44.8 a	116.4 ± 38.4 a	22.2 ± 29.7 a	138.8 ± 41.3 a	86.7 ± 19.7 c
	JAL	HGBS	−42.8 ± 30.5 a	−61.7 ± 9.7 a	122.3 ± 15.3 a	226.7 ± 36.0 a	−57.1 ± 14.1 a	132.9 ± 12.2 a	57.7 ± 12.5 a
		LGBS	−61.0 ± 11.0 a	−69.4 ± 10.4 a	211.9 ± 42.7 a	1122.6 ± 394.2 b	−66.9 ± 10.3 a	236.7 ± 39.0 a	54.0 ± 13.4 a
		HSIN	−36.8 ± 21.7 a	−73.5 ± 10.6 a	130.2 ± 47.4 a	179.1 ± 54.1 a	−63.9 ± 7.9 a	132.7 ± 40.0 a	21.4 ± 38.0 a
		LSIN	−79.1 ± 4.0 a	−44.3 ± 38.7 a	207.8 ± 68.4 a	316.0 ± 130.4 a	−62.3 ± 20.0 a	221.2 ± 76.8 a	94.5 ± 51.2 a
	SA	HGBS	−17.6 ± 17.0 a	−32.6 ± 6.5 ac	−17.8 ± 15.6 a	40.7 ± 33.2 a	−29.0 ± 7.5 a	−8.9 ± 9.7 a	−16.9 ± 6.3 a
		LGBS	−6.2 ± 19.6 a	−14.7 ± 5.3 ab	−12.7 ± 30.1 a	128.8 ± 48.3 a	−9.1 ± 3.8 a	−4.3 ± 28.0 a	−7.2 ± 13.1 a
		HSIN	−31.2 ± 12.8 a	1.7 ± 10.0 b	21.2 ± 17.9 a	−8.1 ± 23.5 a	−8.3 ± 10.0 a	17.2 ± 17.0 a	2.4 ± 9.7 a
		LSIN	−13.2 ± 16.9 a	−45.7 ± 12.6 c	9.4 ± 17.7 a	189.6 ± 110.3 a	−28.9 ± 6.0 a	38.4 ± 13.8 a	8.3 ± 9.0 a
	SAL	HGBS	−34.6 ± 14.5 ab	−64.5 ± 11.7 a	13.6 ± 11.5 a	68.6 ± 42.5 a	−59.2 ± 10.4 ac	19.4 ± 10.9 a	−10.9 ± 10.2 a
		LGBS	−12.5 ± 11.8 a	−35.7 ± 11.2 bc	121.8 ± 44.2 a	685.7 ± 162.8 b	−28.5 ± 9.1 b	138.2 ± 47.0 b	37.9 ± 23.5 a
		HSIN	−57.4 ± 13.9 b	−40.6 ± 4.9 ab	124.7 ± 24.3 a	124.3 ± 62.2 a	−42.9 ± 6.3 ab	123.9 ± 21.7 b	27.4 ± 11.8 a
		LSIN	−68.8 ± 7.9 b	−75.4 ± 6.0 c	91.1 ± 28.6 a	193.3 ± 93.5 a	−71.4 ± 3.0 c	10.6 ± 27.3 ab	26.8 ± 16.0 a
	CL	HGBS	−70.1 ± 7.3 a	−80.7 ± 1.0 a	24.3 ± 14.3 a	71.5 ± 46.4 a	−63.6 ± 2.2 a	30.2 ± 14.4 a	−6.9 ± 7.9 a
		LGBS	−40.4 ± 11.1 a	−83.3 ± 3.3 a	117.6 ± 42.6 a	1697.2 ± 577.7 b	−59.8 ± 5.7 a	170.0 ± 50.0 a	31.7 ± 20.9 a
		HSIN	−59.2 ± 8.0 a	−78.2 ± 3.2 a	97.5 ± 33.0 a	437.9 ± 103.4 a	−52.3 ± 7.0 a	138.7 ± 37.2 a	28.2 ± 14.5 a
		LSIN	−1.0 ± 20.4 b	−67.8 ± 17.1 a	2.6 ± 53.8 a	370.9 ± 206.5 a	−7.8 ± 29.0 b	63.3 ± 48.1 a	31.5 ± 35.7 a

2.2. Glucosinolate Induction over Time after Phytohormone and Herbivory Treatments

2.2.1. Control Plants

For control plants of the HGBS, LGBS, and HSIN genotypes significant differences across times were found for IN (p-values in Table S2). IN contents were highest 9 days after treatments began (Table S3, Figure 2A). In plants of the HSIN genotype TO contents were higher after 3 and 9 days than after 1 day. For plants of the LSIN genotype no significant differences across times were found for the major glucosinolate groups. AL contents did not significantly change through the time of the experiment for any of the four genotypes.

(**A**)



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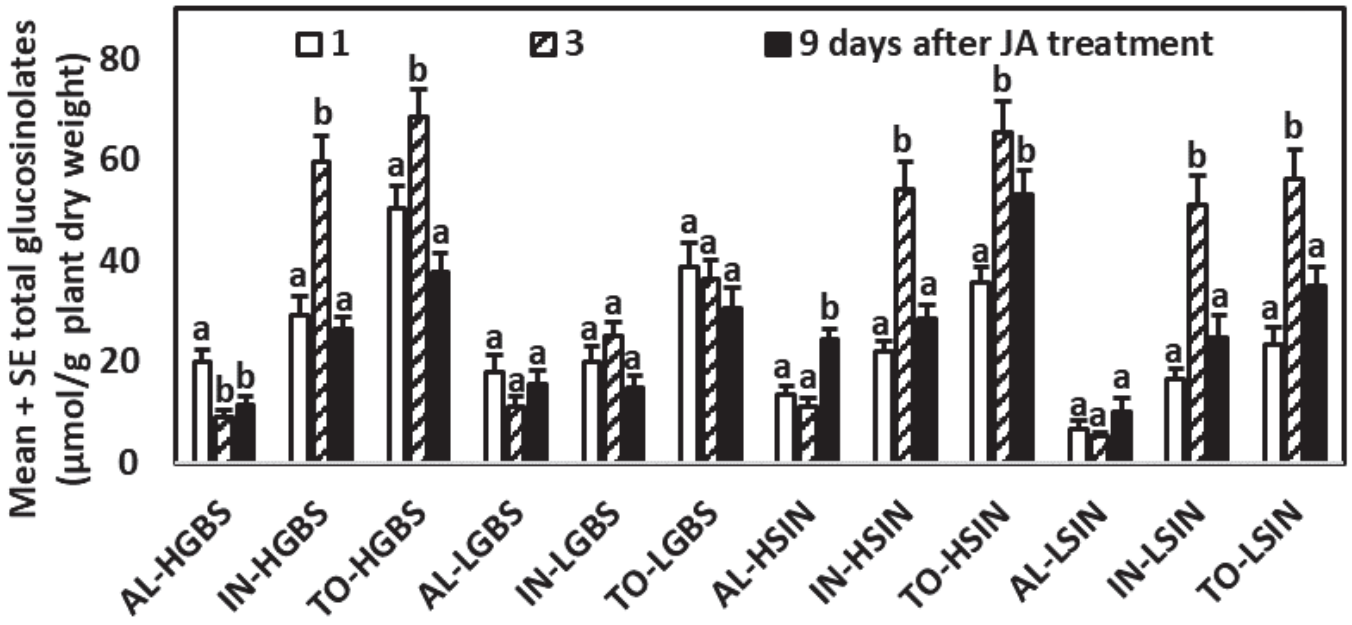

Figure 2. *Cont*.

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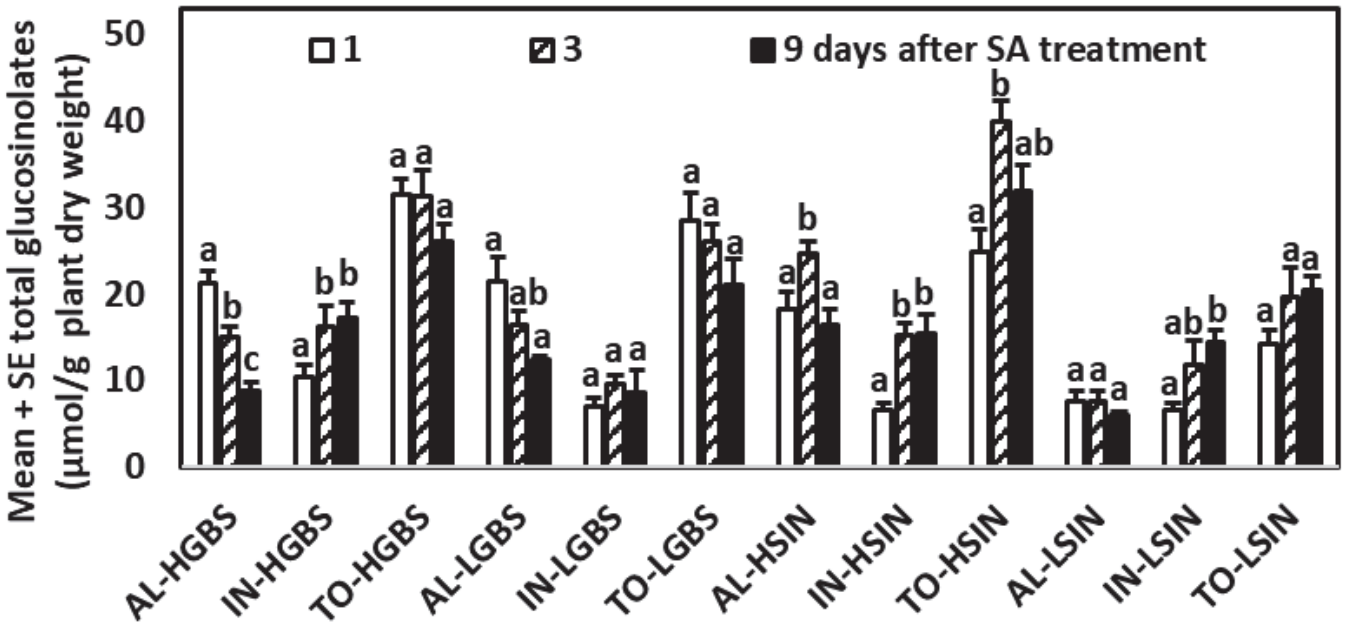

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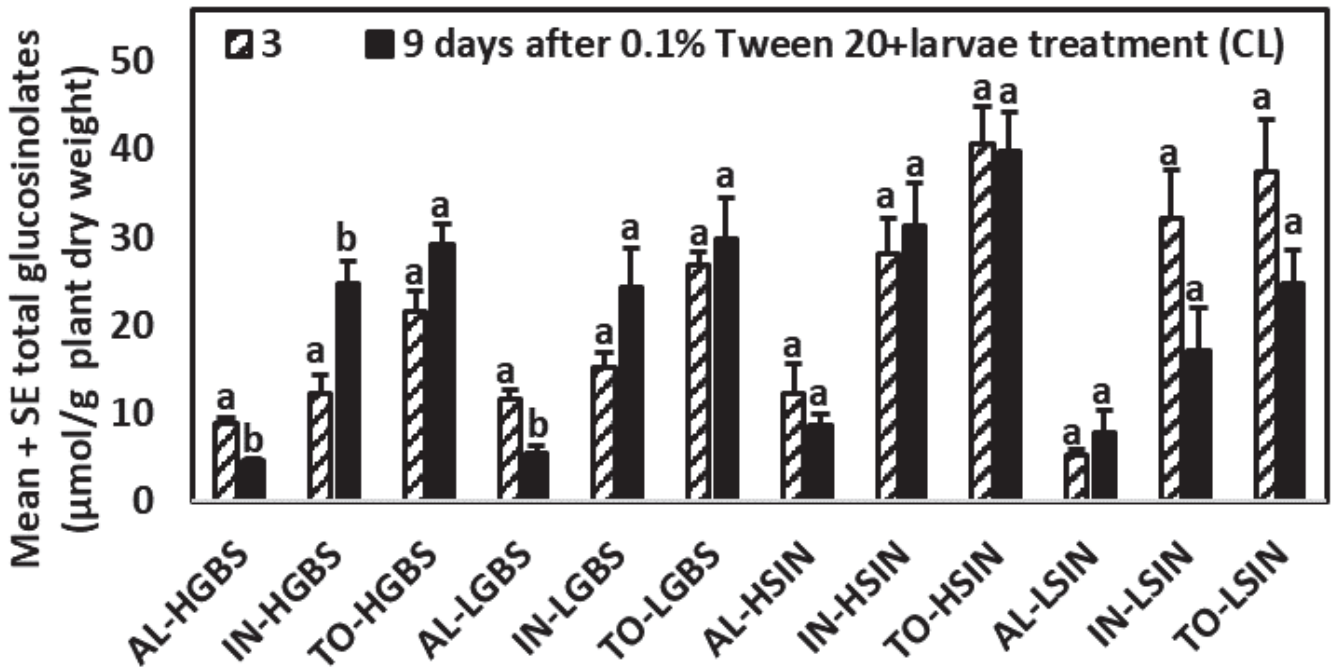

Figure 2. *Cont*.

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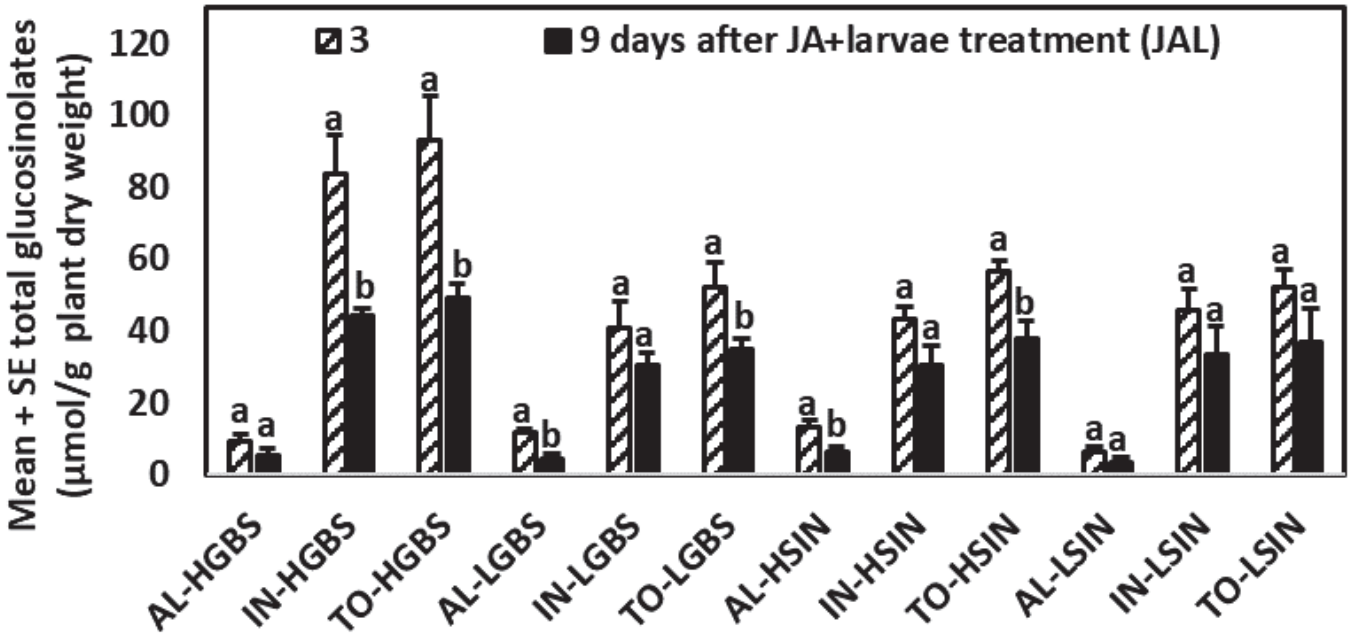


(**F**)

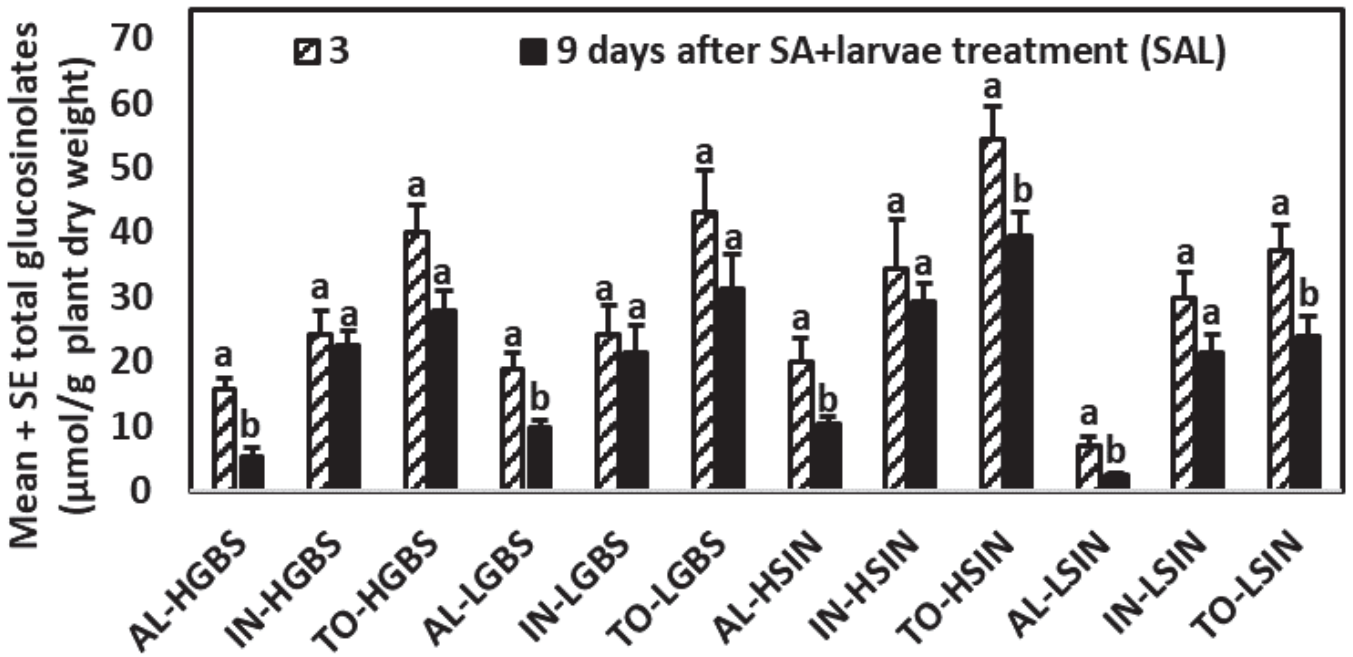


Figure 2. Mean ± SE glucosinolate content (µmol g^{-1} plant dry weight) in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN) 1, 3, and 9 days after treatment, respectively. The treatments are control (C) (**A**), jasmonic acid (JA) (**B**), salicylic acid (SA) (**C**), control with larvae (CL) (**D**), JA with larvae (JAL) (**E**), and SA with larvae (SAL) (**F**). The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among times after treatment within subgroups of total glucosinolates and genotype. Significant differences are shown with different lowercase letters.

2.2.2. Plants Treated with JA

For plants of the HGBS and HSIN genotypes, significant differences across times were found for AL, IN, and TO (Tables S2 and S3; Figure 2B). Concentrations of AL were highest 1 and 9 days after treatment for plants of the HGBS and HSIN genotypes, respectively. For IN and TO, concentrations were highest 3 days after treatment. For plants of the LGBS genotype, differences across times were not significant for the main glucosinolates. For plants of the LSIN genotype, significant differences across times were found for IN and TO, which contents were highest after 3 days. Thus, for JA-treated plants of the HGBS, HSIN, and LSIN genotypes, IN and TO contents were highest 3 days after treatment.

2.2.3. Plants Treated with SA

Significant differences across times for AL were found for all genotypes, except LSIN. Significant differences across times for IN were found for all genotypes, except LGBS. For plants of the HGBS genotype significant differences across times were found for AL and IN (Tables S2 and S3; Figure 2C). For plants of the LGBS genotype significant differences across times were found for AL, which contents were higher 1 day than 9 days after treatment. For plants of the HSIN genotype significant differences across times were found for AL, IN, and TO. For plants of the LSIN genotype significant differences across times were found for IN. SA-treated plants of the HGBS and LGBS genotypes showed the highest AL contents 1 day after treatment, while in plants of the HSIN genotype, AL contents were highest 3 days after treatment. SA-treated plants of the HGBS and LSIN genotypes had in common that IN were highest 9 days after treatment. However, in the case of the HSIN genotype, IN contents did not differ 3 and 9 days after treatment but were higher than 1 day after treatment.

2.2.4. Control Plants with Larval Herbivory (CL Treatment)

For plants of the HGBS genotype, significant differences across times were found for AL and IN (Tables S2 and S3; Figure 2D). For this genotype, concentrations of AL were higher 3 days after the experiment began (2 days after larval feeding started) than 9 days after the experiment began (8 days after larval feeding started). The opposite was found for IN, which concentrations were lower 3 days than 9 days after the experiment began. For plants of the LGBS genotype, significant differences across times were found for AL, which concentrations were also higher 3 days than 9 days after the experiment began. Thus, for CL plants, in both HGBS and LGBS genotypes, AL contents were highest 3 days after the experiment began. For plants of the HSIN and LSIN genotypes, differences across times were not significant for the main glucosinolates.

2.2.5. Plants Treated with JA and Larval Herbivory (JAL Treatment)

For plants of the HGBS genotype, significant differences across times were found for IN and TO (Tables S2 and S3, Figure 2E), which concentrations were highest 3 days after the experiment began. HGBS was the only genotype in which IN content was significantly induced after JAL treatment. JAL plants of the LGBS and HSIN genotypes both had AL and TO contents that were higher 3 days than 9 days after the experiment began. In plants of the HGBS genotype, TO contents were also higher 3 days than 9 days after the experiment began. For plants of the LSIN genotype, no significant differences across times were found for the main glucosinolate groups.

2.2.6. Plants Treated with SA and Larval Herbivory (SAL Treatment)

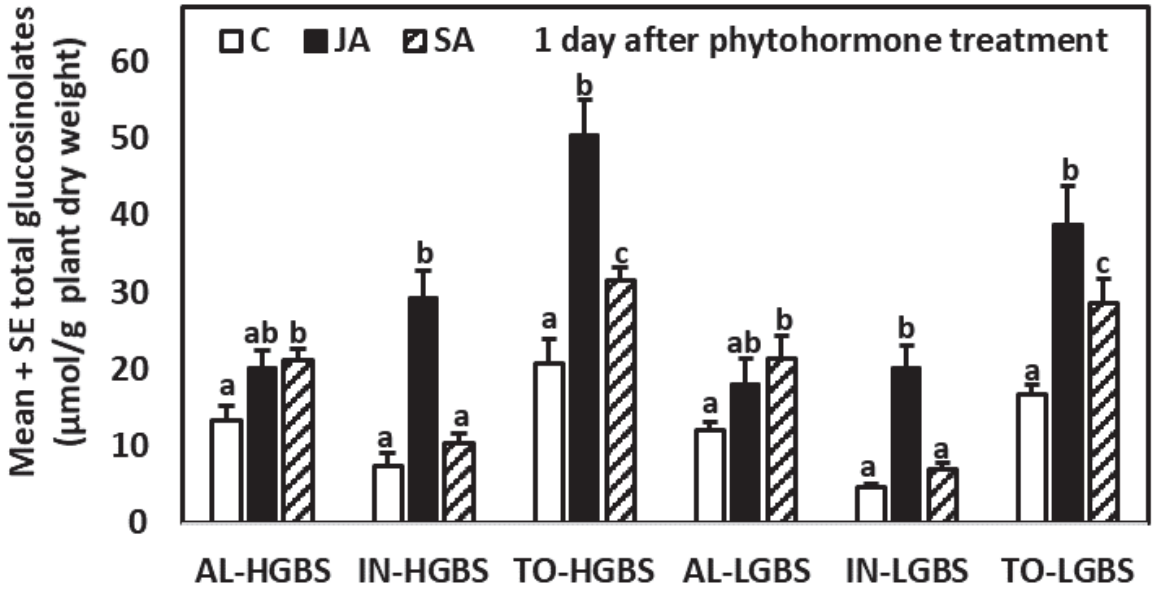
For plants of the four genotypes, significant differences across times were found for AL (Tables S2 and S3; Figure 2F), which concentrations were higher 3 days than 9 days after the experiment began. This highest content 3 days after the experiment began also occurred for TO in the HSIN and LSIN genotypes.

2.3. Differences in Glucosinolate Induction among Treatments and Kale Genotypes: Effect of Phytohormone and Herbivory Treatments

2.3.1. One Day after the Application of Phytohormones

One day after the application of phytohormones, plants of all genotypes showed significant differences among treatments for IN and TO (*p*-values in Table S4) (Table S5, Figure 3). Plants of the HGBS and LGBS genotypes also showed significant differences between treatments for AL, but these differences were not significant in plants of the HSIN and LSIN genotypes. Plants treated with JA had higher content of IN and TO than plants in the control and SA treatments in the four genotypes tested. In plants of the HGBS and LGBS genotypes, AL and TO contents were higher in SA-treated than in control plants, but these differences were not significant in plants of the HSIN and LSIN genotypes.

(**A**)



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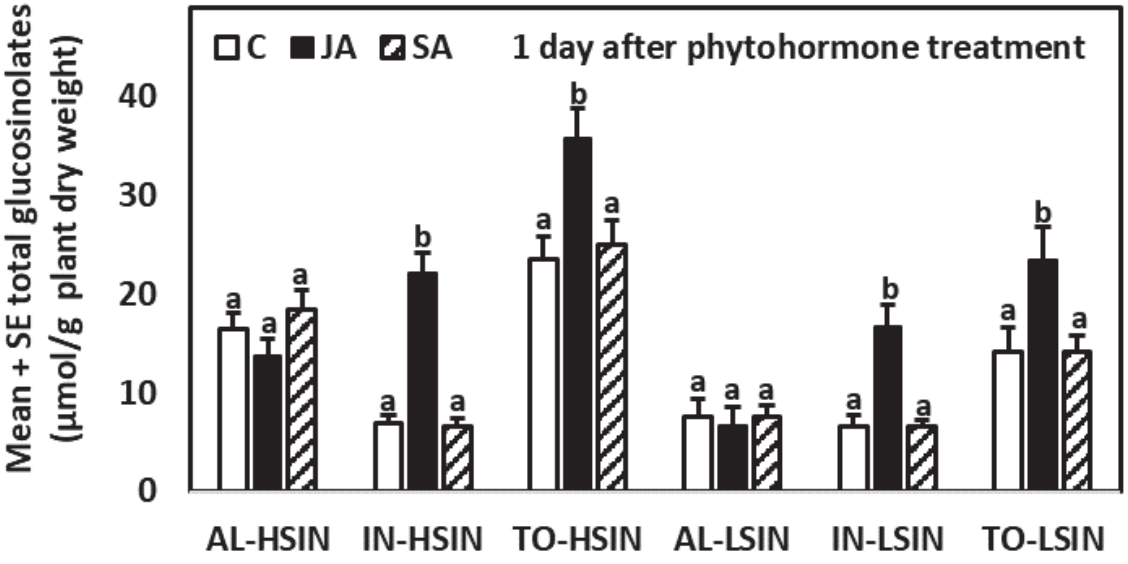


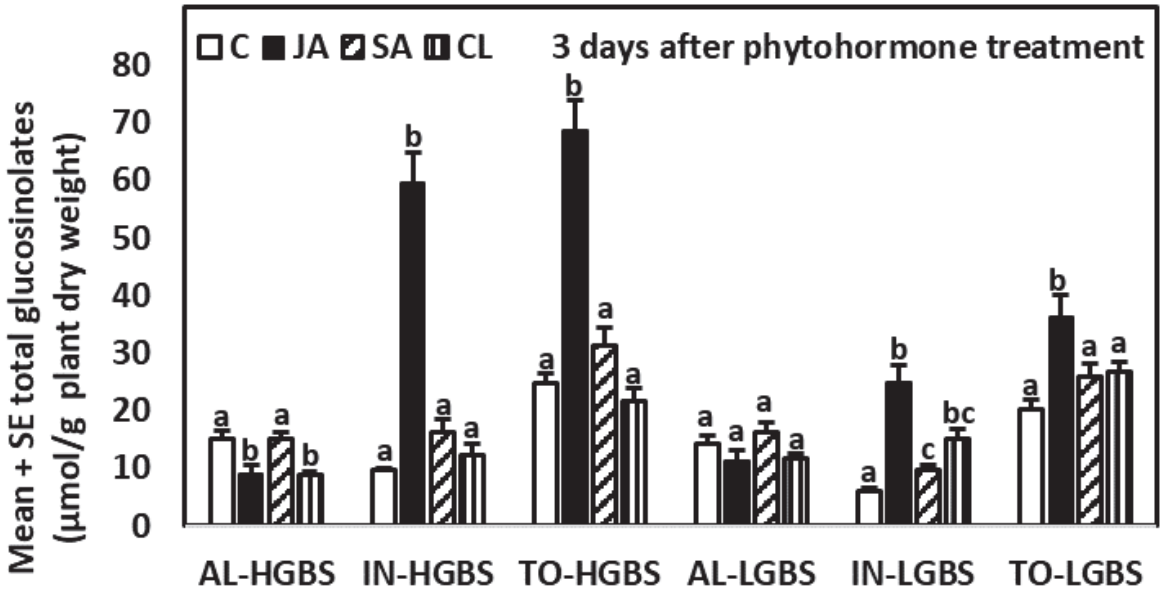
Figure 3. Mean ± SE glucosinolate content (µmol g^{-1} plant dry weight) in kale genotypes high in glucobrassicin (HGBS)

and low in glucobrassicin (LGBS) (**A**), and high in sinigrin (HSIN) and low in sinigrin (LSIN) (**B**). Data shown are from plants one day after application of phytohormones. The treatments are jasmonic acid (JA), salicylic acid (SA), and control (C). The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among phytohormone treatments within subgroups of total glucosinolates and genotype. Significant differences are shown with different lowercase letters.

2.3.2. Three Days after the Application of Phytohormones

Three days after the application of JA and SA, IN and TO contents continued to be higher in JA-treated plants than in plants treated with SA and control plants (Tables S4 and S5; Figure 4). JA treatment increased IN and decreased AL contents in plants of the high glucosinolate genotypes HGBS and HSIN. In contrast, the SA treatment had no significant effect on glucosinolates in plants of any of the genotypes.

(**A**)



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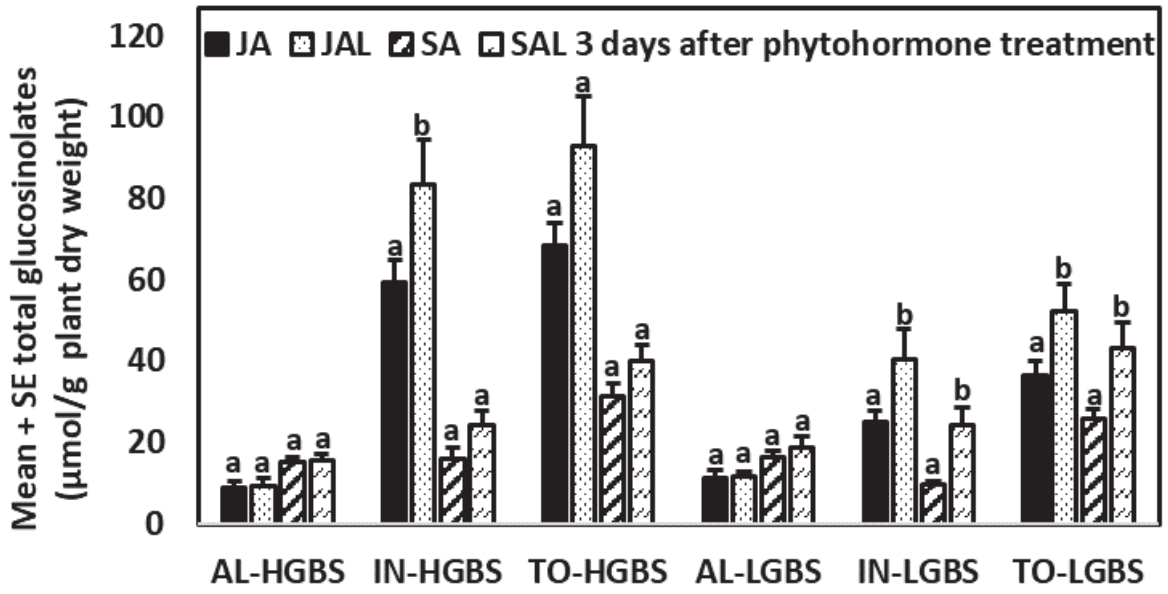
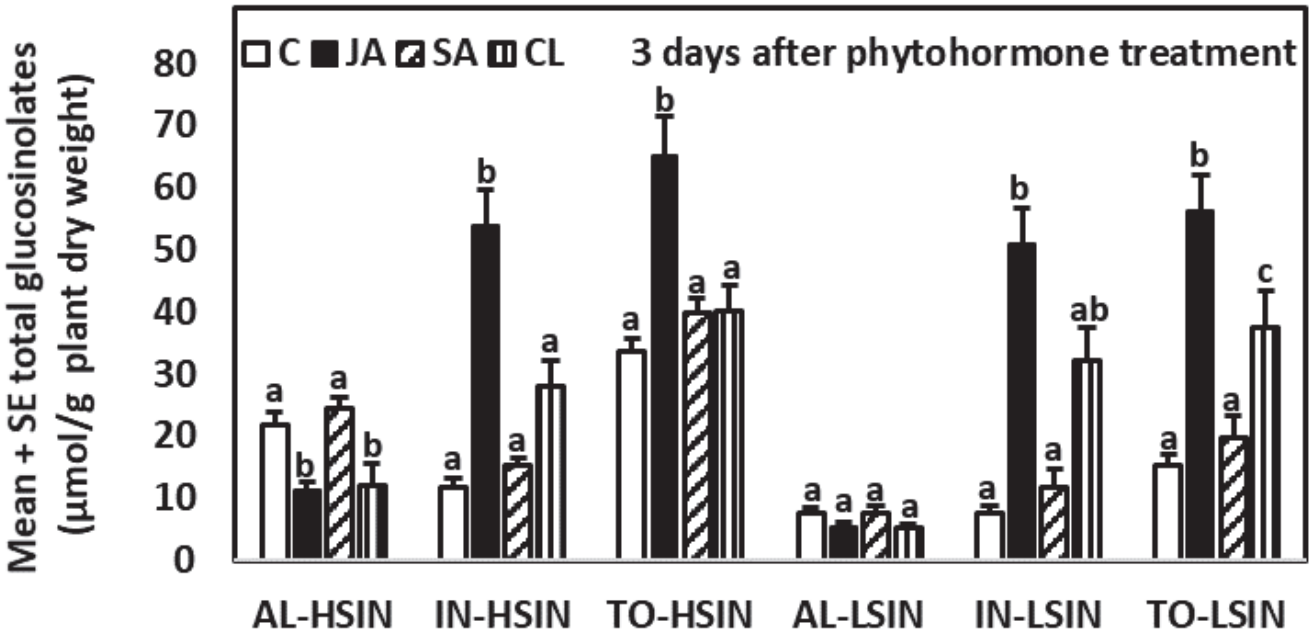


Figure 4. *Cont*.

(**C**)



(**D**)

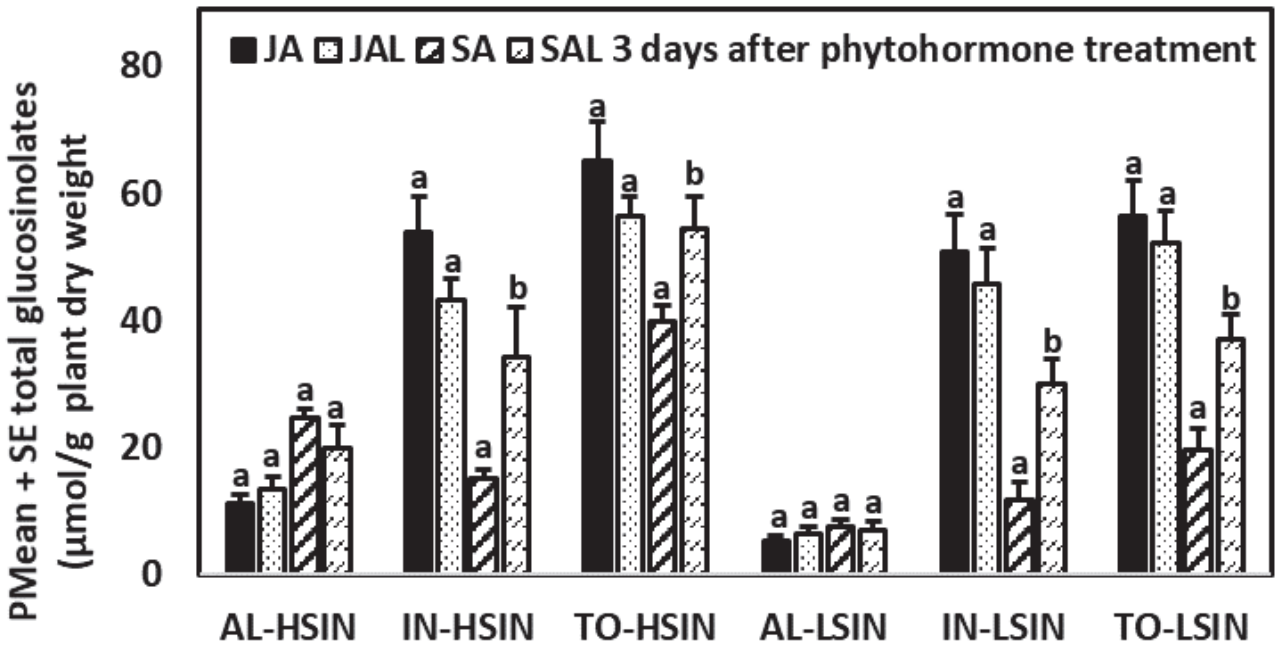


Figure 4. Mean ± SE glucosinolate content (µmol g^{-1} plant dry weight) in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS) (**A**,**B**), high in sinigrin (HSIN), and low in sinigrin (LSIN) (**C**,**D**). Data shown are from plants three days after application of phytohormones. The treatments are jasmonic acid (JA), salicylic acid (SA), JA with larvae (JAL), SA with larvae (SAL), control (C), and control with larvae (CL). The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among phytohormone treatments within subgroups of total glucosinolates and genotype. Significant differences are shown with different lowercase letters.

Herbivory (CL treatment) had a clear effect on the variation of glucosinolates with respect to the control plants. It caused a decrease in AL content in the high glucosinolate genotypes (HSIN and HGBS), while plants in the LSIN genotype showed increased TO

content. An increase in IN content was observed in the LGBS genotype. Compared to JA-treated plants, CL plants had similar levels of AL and lower content of TO in all genotypes. Thus, overall, induction of TO was greater in JA-treated plants than in plants with only larval herbivory. Compared to SA-treated plants, CL plants had lower content of AL in the high glucosinolate genotypes HGBS and HSIN, and higher content of TO in the LSIN genotype.

When comparing JAL and SAL plants (plants of the JA and SA treatments combined with *M. brassicae* larvae), differences in IN and TO content became non-significant as a result of larval feeding, except in the HGBS genotype, in which JAL plants had higher IN and TO content than SAL plants (Tables S4 and S6; Figure 4). Plants from the JAL treatment had higher IN content than JA-treated plants in the HGBS and LGBS genotypes selected for content of the indolic glucosinolate GBS, but these differences were not significant in the case of the HSIN and LSIN genotypes selected for the aliphatic glucosinolate SIN. Except for the HGBS genotype, in which differences were not significant, plants from the SAL treatment had higher contents of IN and TO than SA-treated plants. Therefore, overall, in plants previously treated by JA and SA, larval herbivory increased IN content 3 days after treatment, although this depended on the treatment and genotype.

2.3.3. Nine Days after the Application of Phytohormones

Nine days after the application of phytohormones, IN content continued to be higher in JA-treated plants than in control plants in all genotypes, except LGBS (Tables S4 and S5, Figure 5). JA-treated plants also had higher content of TO content than control plants in the HSIN and LSIN genotypes. JA-treated plants of the LSIN genotype also had higher content of AL than control plants. Differences in glucosinolate content between JA- and SA-treated plants and between control and SA-treated plants were no longer significant.

(**A**)

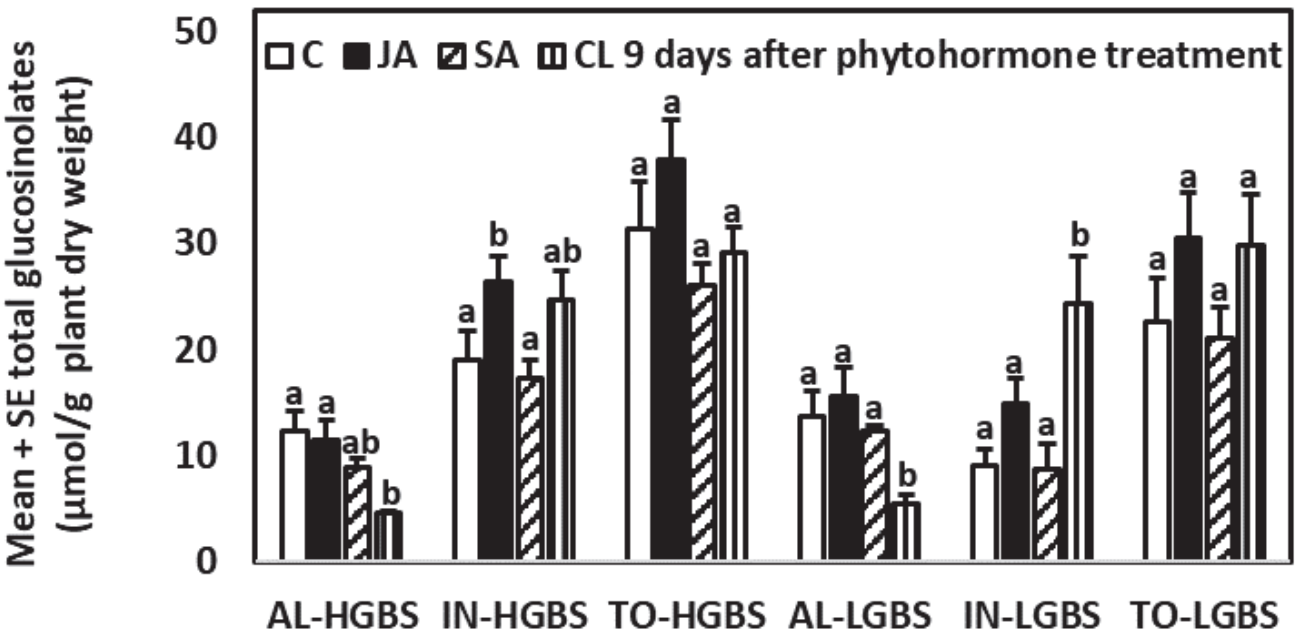
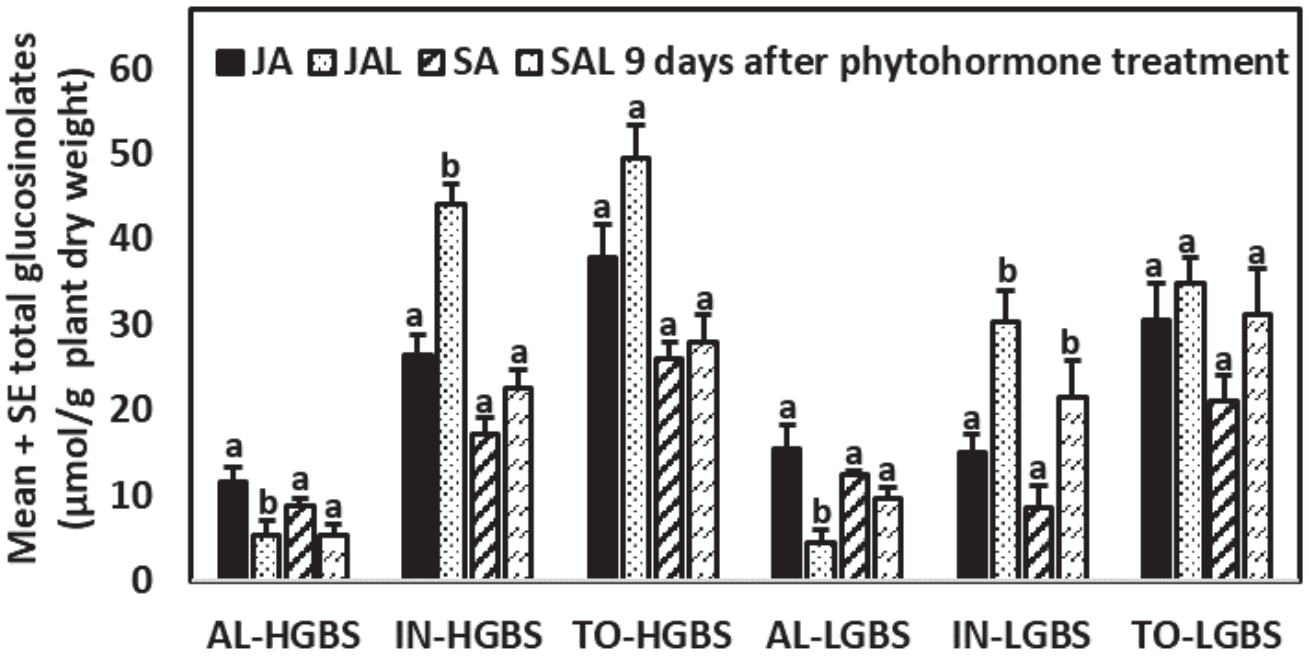


Figure 5. *Cont*.

(**B**)



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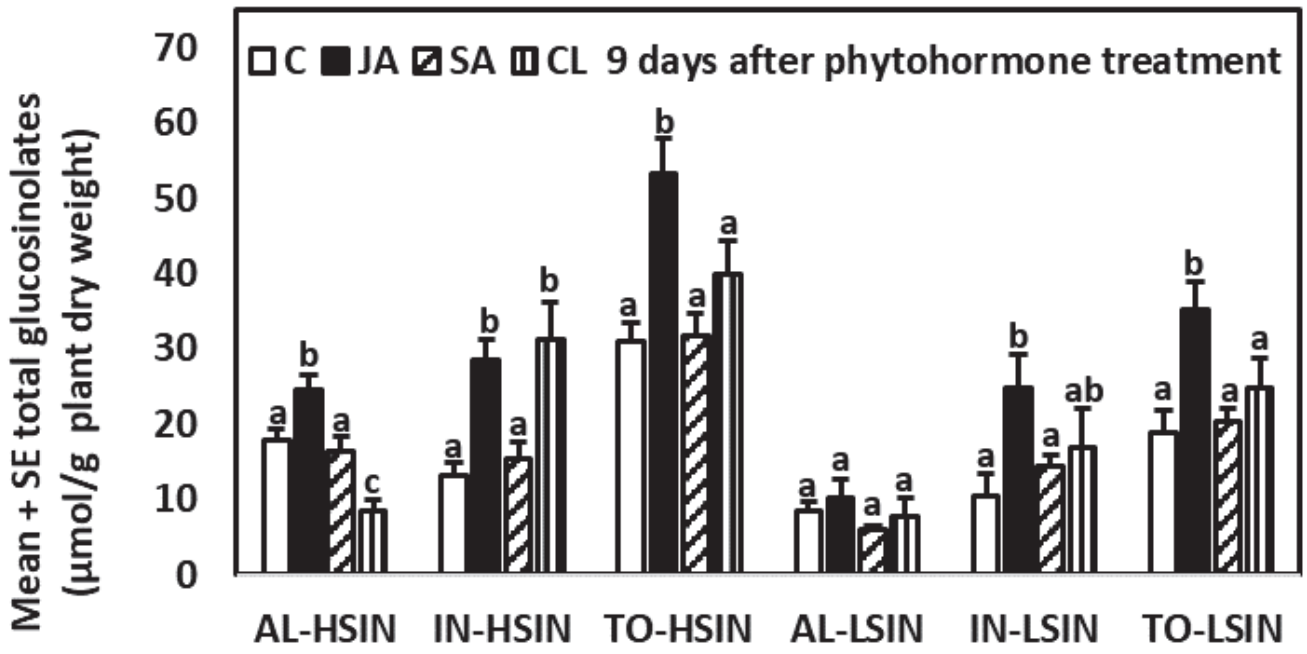


Figure 5. *Cont*.

(**D**)

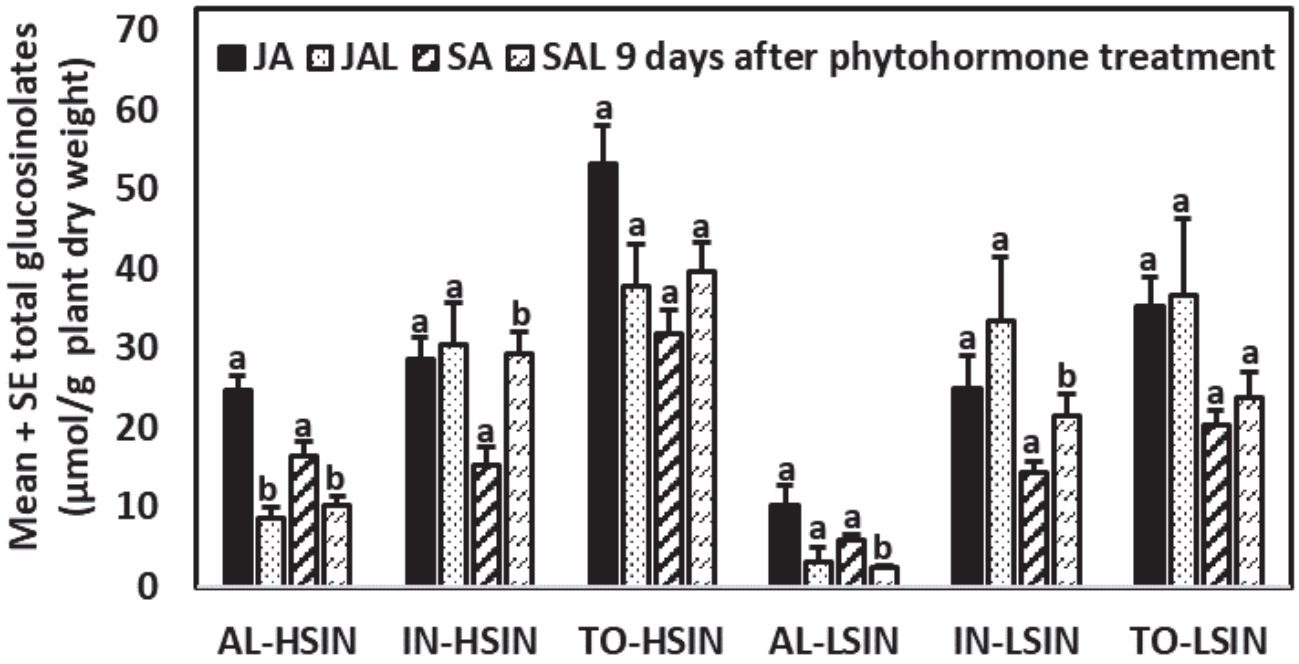


Figure 5. Mean ± SE glucosinolate content (µmol g^{-1} plant dry weight) in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS) (**A**,**B**), high in sinigrin (HSIN), and low in sinigrin (LSIN) (**C**,**D**). Data shown are from plants nine days after application of phytohormones. The treatments are jasmonic acid (JA), salicylic acid (SA), JA with larvae (JAL), SA with larvae (SAL), control (C), and control with larvae (CL). The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among phytohormone treatments within subgroups of total glucosinolates and genotype. Significant differences are shown with different lowercase letters.

The CL treatment had lower content of AL than control and JA-treated plants without larvae, except in the LSIN genotype, in which these differences were not significant. Plants in the CL treatment also had lower content of AL than SA plants of the HSIN and LGBS genotypes, but not in the others. In the LGBS genotype, CL plants had higher content of IN than control plants, JA-treated, and SA-treated plants, but in the other genotypes differences between these treatments were not significant.

Plants from the JAL treatment had lower AL and higher IN contents than JA-treated plants of the HGBS and LGBS genotypes (Tables S4 and S6; Figure 5). Plants from the JAL treatment also had lower content of AL than JA-treated plants of the HSIN treatment. Plants from the SAL treatment had lower AL and higher IN contents than SA-treated plants of the HSIN and LSIN genotypes. Plants from the SAL treatment also had higher content of IN than SA-treated plants of the LGBS treatment. There were no significant differences in TO content between plants JA and JAL treatments nor between plants of the SA and SAL treatments. Therefore, overall, in plants previously treated with JA and SA, larval herbivory decreased and increased AL and IN contents, respectively, although this depended on the treatment and genotype.

2.4. Differences in Glucosinolate Induction among Treatments and Kale Genotypes: Glucosinolate Differences among Genotypes

2.4.1. One Day after the Application of Phytohormones

In control plants, there were significant differences among genotypes for AL and TO (*p*-values in Table S7) (Table S8). AL and TO contents were higher in plants of the HSIN genotype than in plants of the LSIN genotype. TO content was also higher in plants of the HSIN genotype than in plants of the LGBS genotype.

In JA-treated plants, there were significant differences among genotypes for AL, IN, and TO, which contents were lowest in plants of the LSIN genotype. In SA-treated plants, there were significant differences among genotypes for AL, IN, and TO. The contents of AL and TO in SA-treated plants were lowest in plants of the LSIN genotype, while IN content was highest in the HGBS genotype. Thus, 1 day after the application of phytohormones, both JA- and SA-treated plants had in common that content of AL and TO were lowest in plants of the LSIN genotype. Compared to plants in the control treatment, in which there were no significant differences in IN content among genotypes, the application of JA and SA resulted in significant differences among genotypes for IN content.

2.4.2. Three Days after the Application of Phytohormones

In control plants, there were significant differences among genotypes for AL, IN, and TO (Tables S7 and S8). AL and TO were highest in plants of the HSIN genotype, while IN contents were higher in plants of the HGBS and HSIN genotypes than in plants of the LGBS genotype.

In JA-treated plants, there were significant differences among genotypes for AL, IN, and TO. Contents of IN and TO were lowest in JA-treated plants of the LGBS genotype, while contents of AL were lowest in the LSIN genotype. In SA-treated plants, there were significant differences among genotypes for AL and TO, which were lowest in the LSIN genotype, but there were no significant differences among genotypes for IN. Thus, compared to plants of the control treatment, in SA-treated plants differences in IN content among genotypes were no longer significant. In the CL treatment, there were significant differences among genotypes for IN and TO, the contents of which were higher in plants of the HSIN and LSIN genotypes than in plants of the HGBS genotype. Content of TO was also higher in CL-treated plants of the HSIN genotype than in CL-treated plants of the LGBS genotype. Compared to the control plants, as a result of larval feeding, IN contents were no longer higher in plants of the genotype HGBS than in plants of the genotype LGBS.

In the JAL treatment, there were significant differences among genotypes for IN and TO. IN and TO contents were highest in the HGBS genotype. In the SAL treatment, there were significant differences among genotypes for AL, which contents were lowest in the LSIN genotype. Compared to the control plants, in JAL- and SAL-treated plants IN contents were no longer higher in plants of the genotype HGBS than in plants of the genotype LGBS. Plants in the JA, SA, and SAL treatments had in common that content of AL continued to be lowest in plants of the LSIN genotype 3 days after the application of phytohormones.

2.4.3. Nine Days after the Application of Phytohormones

In control plants, there were significant differences among genotypes for AL and IN (Tables S7 and S8). AL contents were higher in plants of the HSIN genotype than in plants of the LSIN and HGBS genotypes, while IN contents were higher in plants of the HGBS genotype than in plants of the LSIN and LGBS genotypes.

In both JA- and SA-treated plants, there were significant differences among genotypes for AL, IN, and TO; contents of AL and TO were highest in the HSIN genotype, while content of IN was lowest in the LGBS genotype. Regarding the effect of induction by larval feeding, in plants of the CL and JAL treatments, there were no significant differences among genotypes in the contents of the main glucosinolate groups. In the SAL treatment, there were significant differences among genotypes for AL, which contents were higher in plants of the LGBS and HSIN genotypes than in plants of the HGBS and LSIN genotypes. Compared to the control plants, in CL-, JAL-, and SAL-treated plants, IN contents were no longer higher in plants of the genotype HGBS than in plants of the genotype LGBS; in the case of the CL- and JAL- treated plants, AL contents were no longer higher in plants of the HSIN genotype than in plants of the LSIN and HGBS genotypes.

2.5. Differences in Glucosinolate Induction among Treatments and Kale Genotypes: Percent Glucosinolate Variation among Genotypes

2.5.1. One Day after the Application of Phytohormones

Regarding differences in percent changes in glucosinolate content among genotypes compared to the controls of each genotype, one day after the application of phytohormones there were significant differences among genotypes for AL, IN, TO, GIB, and NEO in both JA and SA treatments (*p*-values in Table S9) (Table 1; Figure 6). In the JA treatment there were also significant differences among genotypes for SIN.

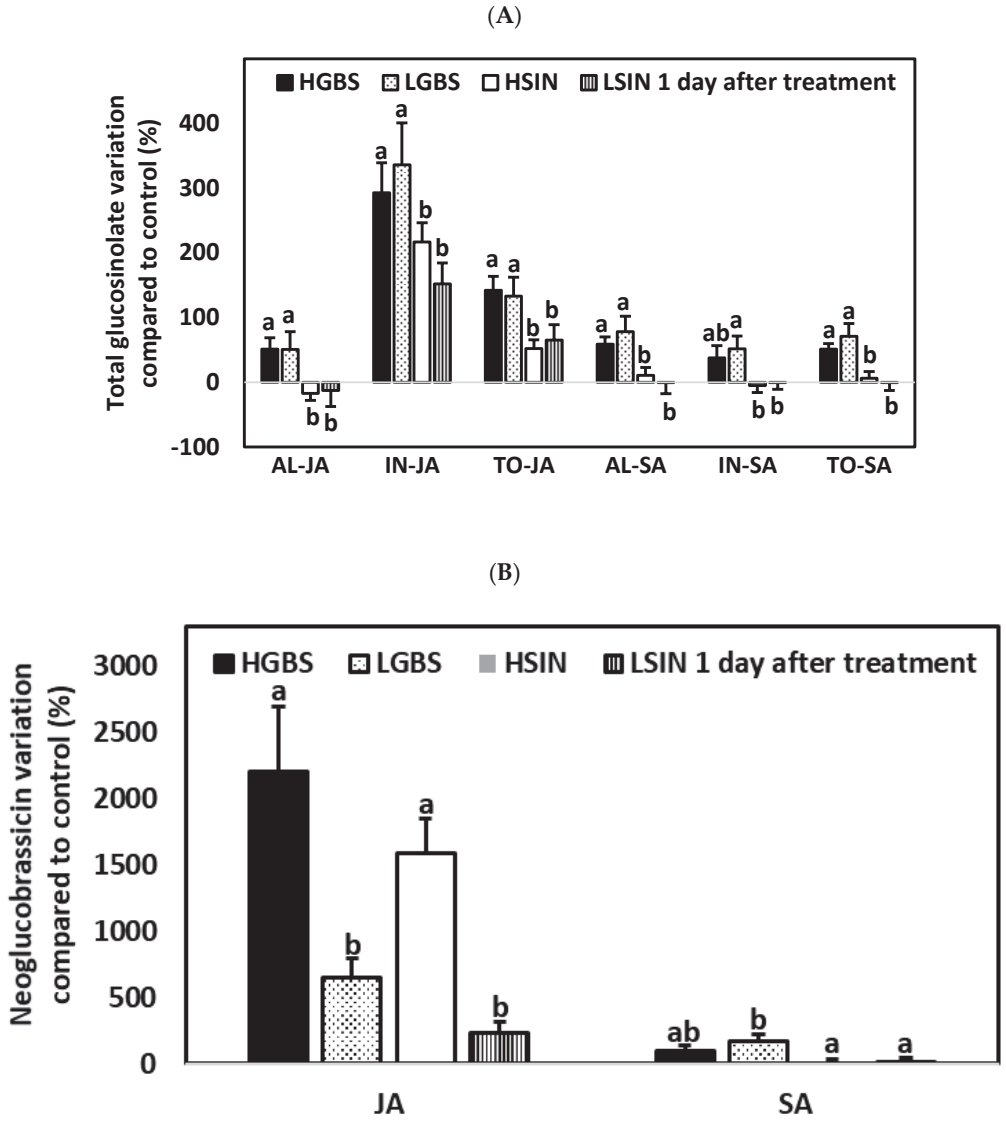


Figure 6. Mean ± SE percentage glucosinolate content related to control plants in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN) one day after treatment with jasmonic acid (JA) and salicylic acid (SA). The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO) (**A**) and neoglucobrassicin (**B**). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among genotypes within subgroups of treatment and glucosinolate content. Significant differences are shown with different lowercase letters.

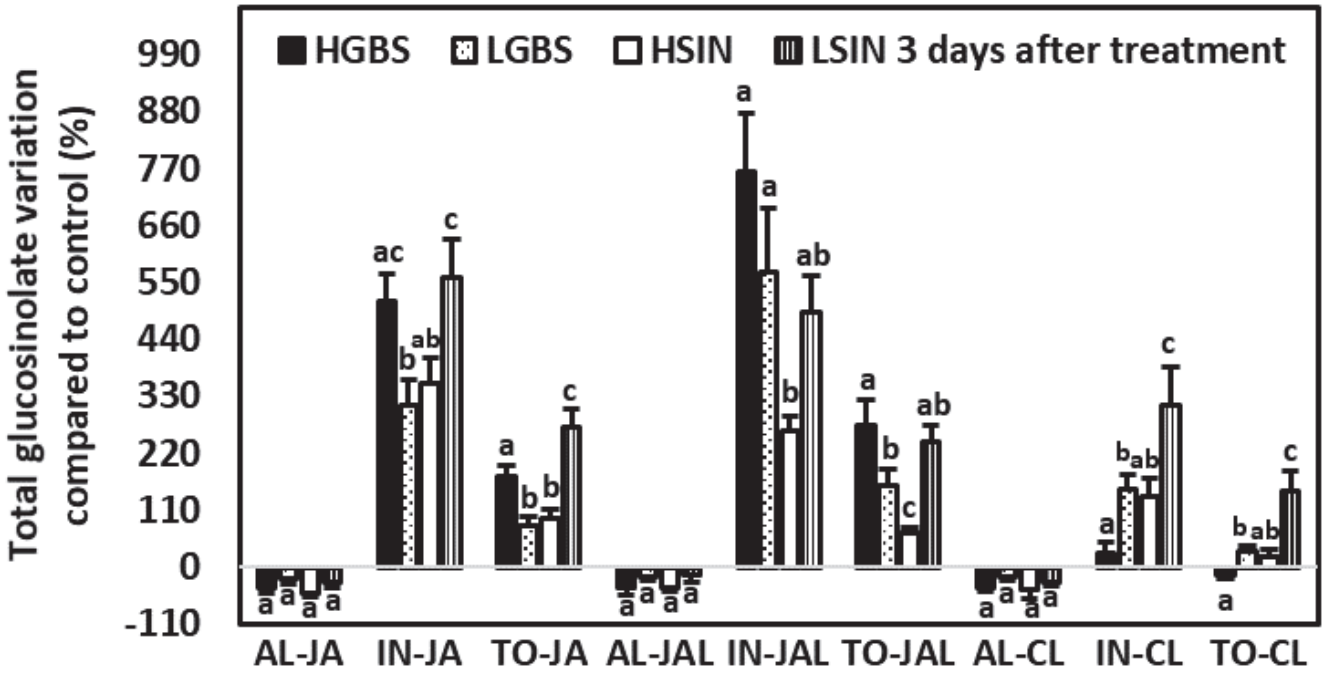
Percent changes in AL and TO contents in JA- and SA-treated plants were higher in plants of the HGBS and LGBS genotypes than in the HSIN and LSIN genotypes (Figure 6A). Changes in glucosinolate content among JA-treated plants of the four plant genotypes were mostly due to an increase in IN contents. In JA-treated plants, IN contents

increased between 151.9% and 335.7%, and the percentage of increase was higher in plants of the genotypes HGBS and LGBS than in plants of the genotype LSIN. The glucosinolate that increased most was NEO, which increased more in the HGBS and HSIN genotypes (2198.0% and 1584.8% increases, respectively) than in the LGBS and LSIN genotypes (Figure 6B). In SA-treated plants, changes in IN contents ranged from a 5% decrease in plants of the HSIN genotype to a 51.6% increase in plants of the LGBS genotype. This percent change in IN content was higher in the genotype LGBS than in the genotypes HSIN and LSIN. As in the case of JA-treated plants, SA-treated plants showed an increase in NEO content, but this increase was not as high as in JA-treated plants. In SA-treated plants, NEO also showed a higher increase in the HGBS and LGBS genotypes (94.5% and 171.8% increases, respectively) than in the LSIN and HSIN genotypes. As a result of JA treatment, the glucosinolate GIB increased more in plants of the genotypes HGBS, BGBS, and HSIN (69.3%, 60.8%, and 24.2% increases, respectively) than in plants of the genotype LSIN, in which this glucosinolate decreased. Changes in glucosinolate content among SA-treated plants in the four plant genotypes were mostly due to the increase in GIB, which increased more in plants of the HGBS and LGBS genotypes (97.2% and 82.6% increases, respectively) than in plants of the HSIN and LSIN genotypes.

2.5.2. Three Days after the Application of Phytohormones

There were no significant percent changes in AL content among the four genotypes in any of the treatments (Table S9). Percent changes in AL content were negative for all genotypes under the JA, JAL, and CL treatments and in the genotypes HSIN and LSIN under the SAL treatment. There were significant differences among genotypes for IN, TO, and NEO in the JA treatment (Tables 1 and S9, Figure 7). In both the SA and SAL treatments, there were no significant differences among genotypes for any of the main glucosinolates. In the CL and JAL treatments there were significant differences among genotypes for IN, TO, and GBS.

(**A**)



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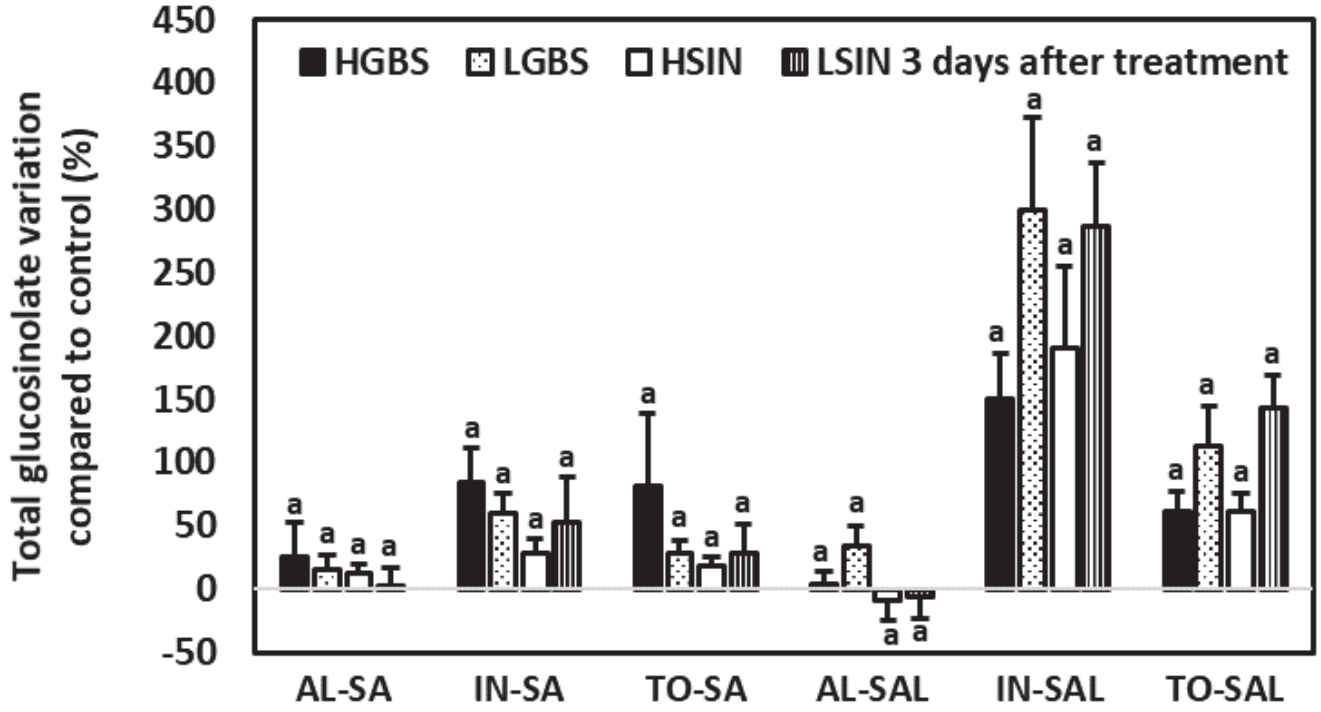


Figure 7. *Cont.*

(**C**)

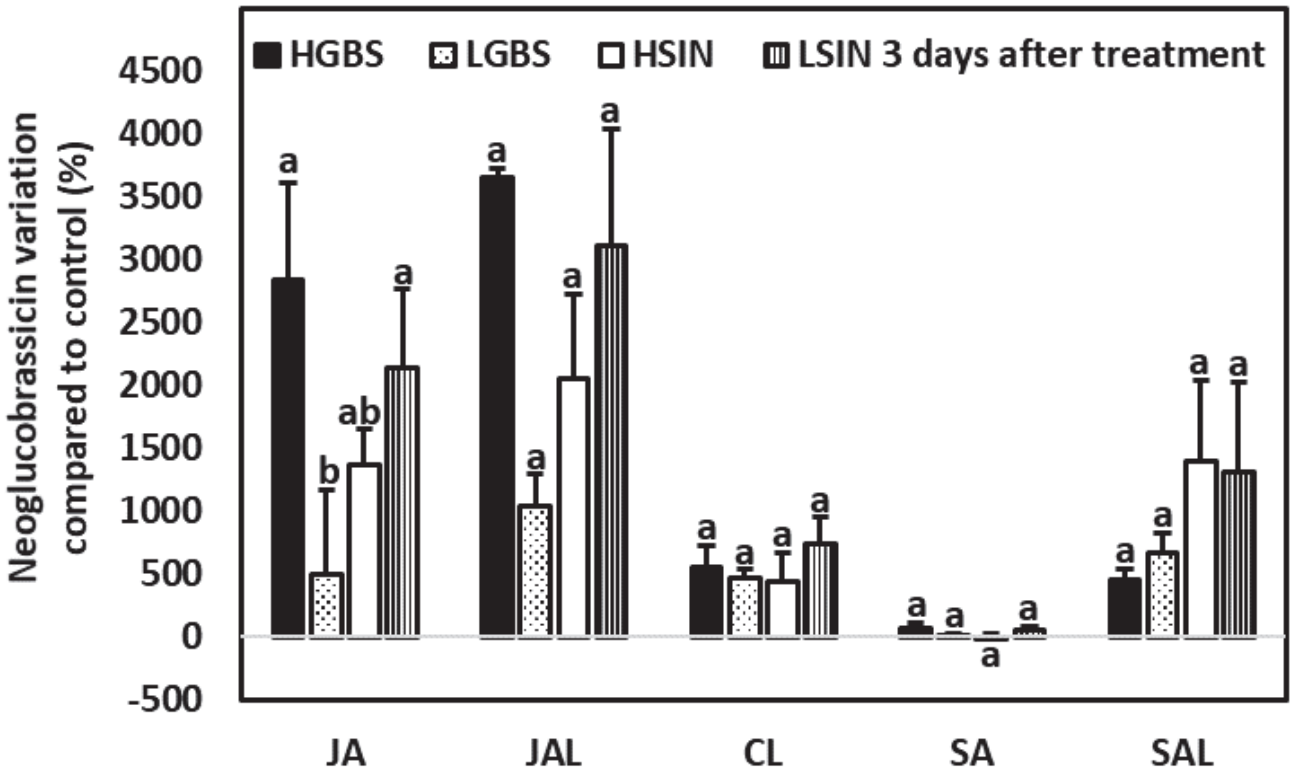


Figure 7. Mean ± SE percentage glucosinolate content related to control plants in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). Data shown are from plants three days after application of phytohormones. The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO) for the treatments with jasmonic acid (JA), JA with larvae (JAL), and control with larvae (CL) (**A**) and after treatment with salicylic acid (SA), and SA with larvae (SAL) (**B**). Additionally, neoglucobrassicin is also shown for the treatments JA, JAL, CL, SA, and SAL (**C**). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among genotypes within subgroups of treatment and glucosinolate content. Significant differences are shown with different lowercase letters.

In JA-treated plants percent changes in IN and TO content were highest in the HGBS and LSIN genotypes. In JA-treated plants IN contents increased between 313.6% in plants of the LGBS genotype and 335.7% in plants of the LSIN genotype. IN induction was mostly due to NEO increase. Percent increases in NEO contents in JA-treated plants were significantly higher in the HGBS and LSIN genotypes than in the LGBS genotype (Tables 1 and S9; Figure 7C). The highest percent increase in NEO content occurred in the HGBS genotype (2837.5%). The percent increases in GBS in the HGBS genotype (355.3%) and in the LGBS genotype (300.4%) were not significantly different.

In plants of the JAL treatment, percent increase in TO contents was lower in the HSIN genotype than in the other genotypes. The percentage increase in IN was higher in the genotypes HGBS and LGBS (763.2% and 570.8%, respectively) than in the genotype HSIN. It was mostly due to NEO variation, ranging from 1039.6% in plants of the LGBS genotype to 3655.5% in plants of the HGBS genotype, but differences among genotypes were not significant. Percent increases in GBS were highest in plants of the HGBS and LGBS genotypes (568.9% and 545.6%, respectively).

In SA- and SAL-treated plants there were no significant differences in percent changes in AL, IN, TO, GIB, SIN, GBS, and NEO contents among the different plant genotypes.

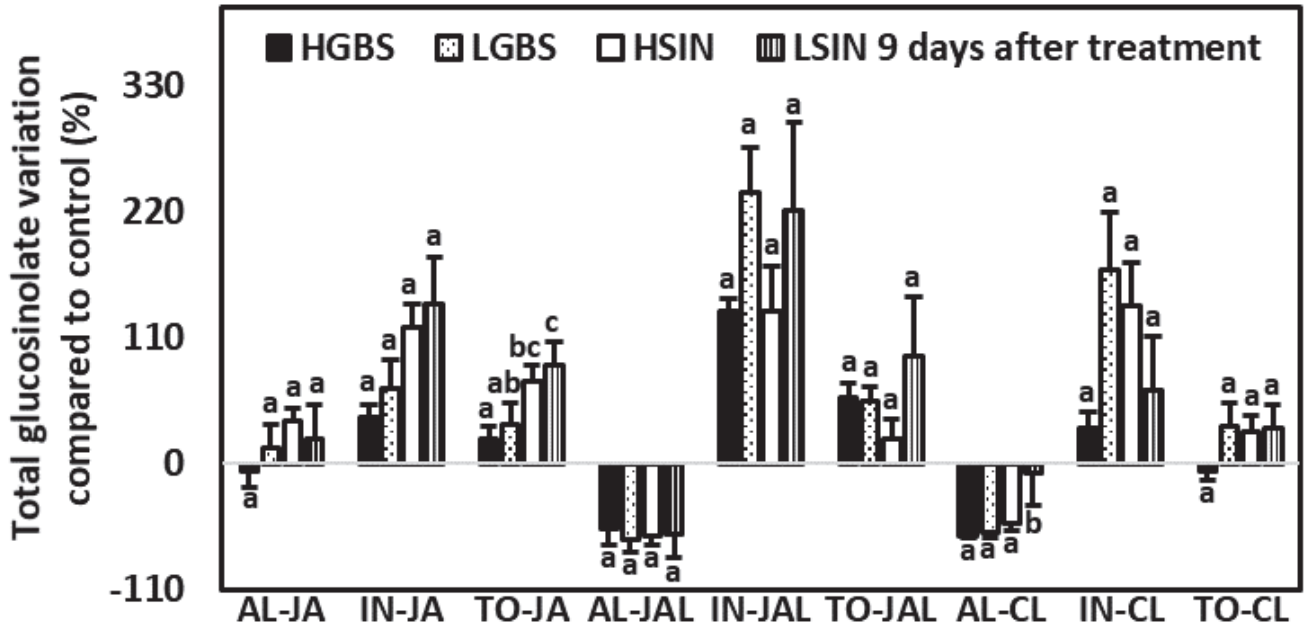
In the CL treatment, percent changes in IN and TO content were higher in plants of the LSIN genotype than in the other genotypes. Among individual glucosinolates, the

percent variation in GBS was highest in plants of the LSIN genotype (268.7%) and lowest in plants of the HGBS genotype, which showed a decrease of −11.6%.

2.5.3. Nine Days after the Application of Phytohormones

There were significant differences among genotypes for TO and NEO in the JA treatment, while in the SA treatment there were only significant differences among genotypes for SIN (Tables 1 and S9, Figure 8). In the CL treatment, there were significant differences among genotypes for AL, GIB, and NEO. In the JAL treatment, there were only significant differences among genotypes for NEO. In the SAL treatment, there were significant differences among genotypes for AL, IN, GIB, SIN, and NEO.

(**A**)



(**B**)

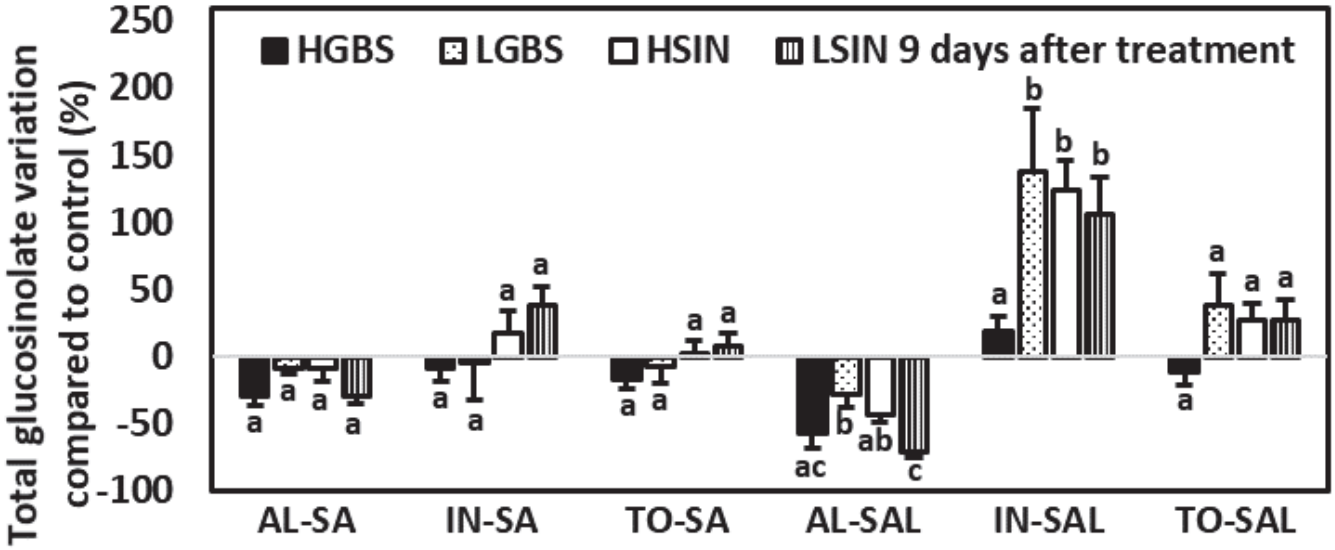


Figure 8. *Cont.*

(**C**)

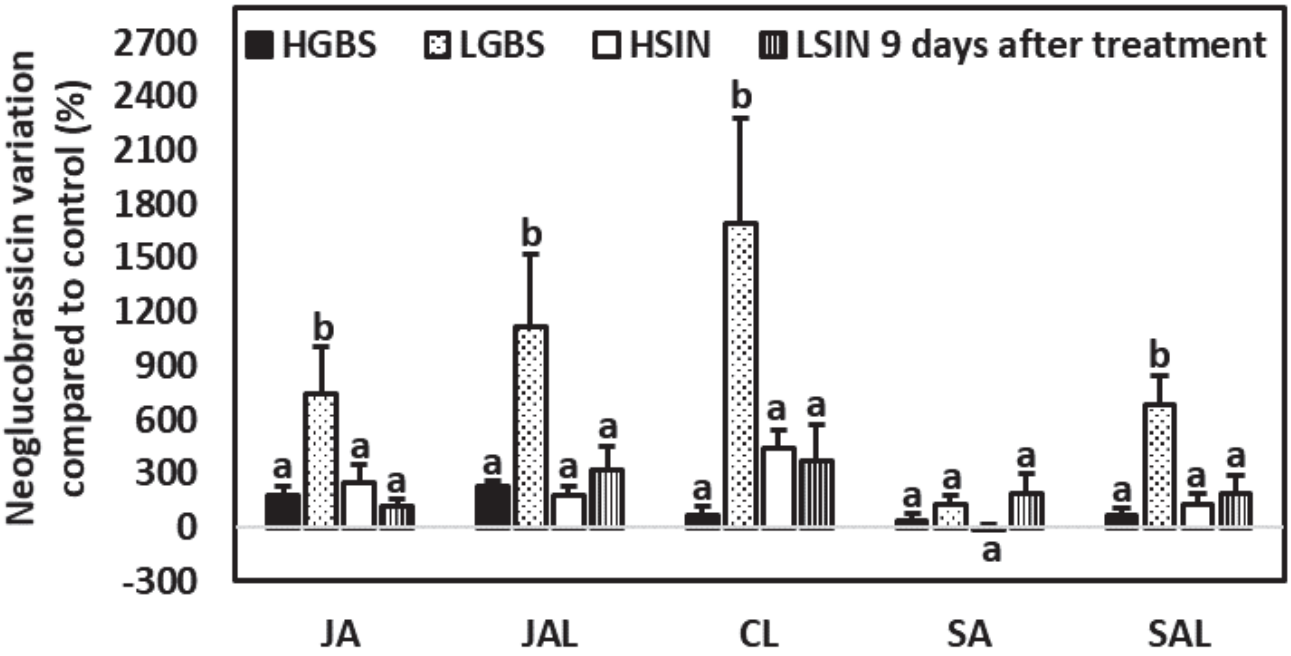


Figure 8. Mean ± SE percentage glucosinolate content related to control plants in kale genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). Data shown are from plants nine days after application of phytohormones. The glucosinolates shown are total aliphatic (AL), total indolic (IN), and total glucosinolates (TO) for the treatments with jasmonic acid (JA), JA with larvae (JAL), and control with larvae (CL) (**A**) and after treatment with salicylic acid (SA), and SA with larvae (SAL) (**B**). Additionally, neoglucobrassicin is also shown for the treatments JA, JAL, CL, SA, and SAL (**C**). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences among genotypes within subgroups of treatment and glucosinolate content. Significant differences are shown with different lowercase letters.

Significant percent changes in TO content among plant genotypes occurred only in JA-treated plants, in which the percentage increase in TO content was highest in the HSIN and LSIN genotypes (71.4% and 86.7%, respectively).

Except in the SAL treatment, there were no significant differences among genotypes in percentage variation of IN, which percent increase was highest in plants of the LGBS and HSIN genotypes (138.2% and 123.9%, respectively).

Significant percentage changes in AL content occurred only in the CL and SAL treatments. In the case of the CL treatment, the percentage decrease in AL was not as much in plants of the LSIN genotype (−7.8%) as in plants of the other genotypes. However, in the case of the SAL treatment, the percentage decrease in AL was highest in plants of the LSIN genotype (−71.4%).

Among individual glucosinolates, significant changes in percentage variation among genotypes were observed in NEO in all treatments except the SA treatment, and these changes in NEO were highest in the LGBS genotype (Tables 1 and S9; Figure 8C). These percent increases in NEO content in plants of the LGBS genotype were 746.9%, 1122.6%, 685.7%, and 1697.2% for the JA, JAL, SAL, and CL treatments, respectively. In the SA and SAL treatments, differences in percentage variation among genotypes were also significant for SIN, being lowest in plants of the LSIN genotype, which plants showed decreases of −45.7% and −75.4% for the SA and SAL treatments, respectively. In the SAL treatment, the percentage decrease in GIB was higher in plants of the HSIN and LSIN genotypes (−57.4% and −68.8%, respectively) than in plants of the LGBS genotype.

2.6. Correlation between Induced Aliphatic and Indolic Glucosinolates

There was a positive correlation between AL and IN content 1 day after JA and SA treatment, 3 days after SA treatment, and 9 days after SAL treatment (Table S10). However, 3 days after treatment, in the CL treatment, there was a negative correlation between AL and IN.

2.7. Herbivory and Larval Weight Gain Experiments

For the genotypes HGBS, HSIN, and LSIN, *M. brassicae* larval weights were lower in JA-treated plants than in SA-treated and control plants (Figure 9; Table S11). Larval weights in the SA and control treatments were not significantly different. In plants of the LGBS genotype, differences in larval weights among treatments were not significantly different.

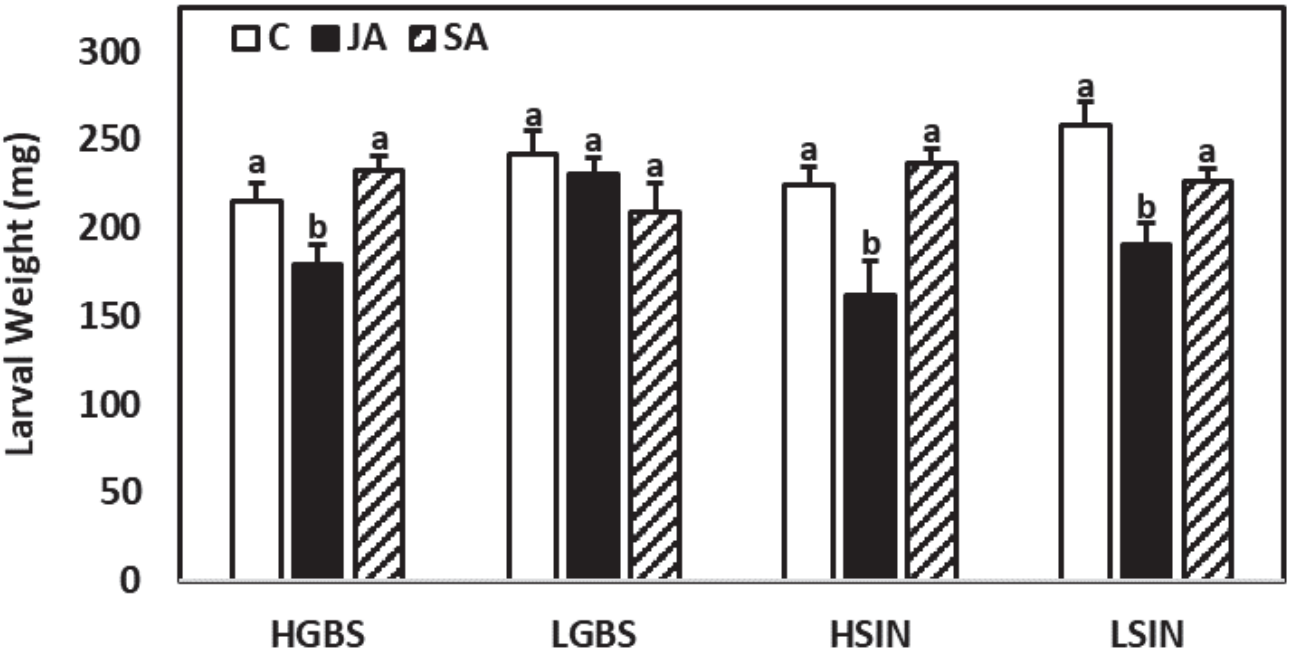
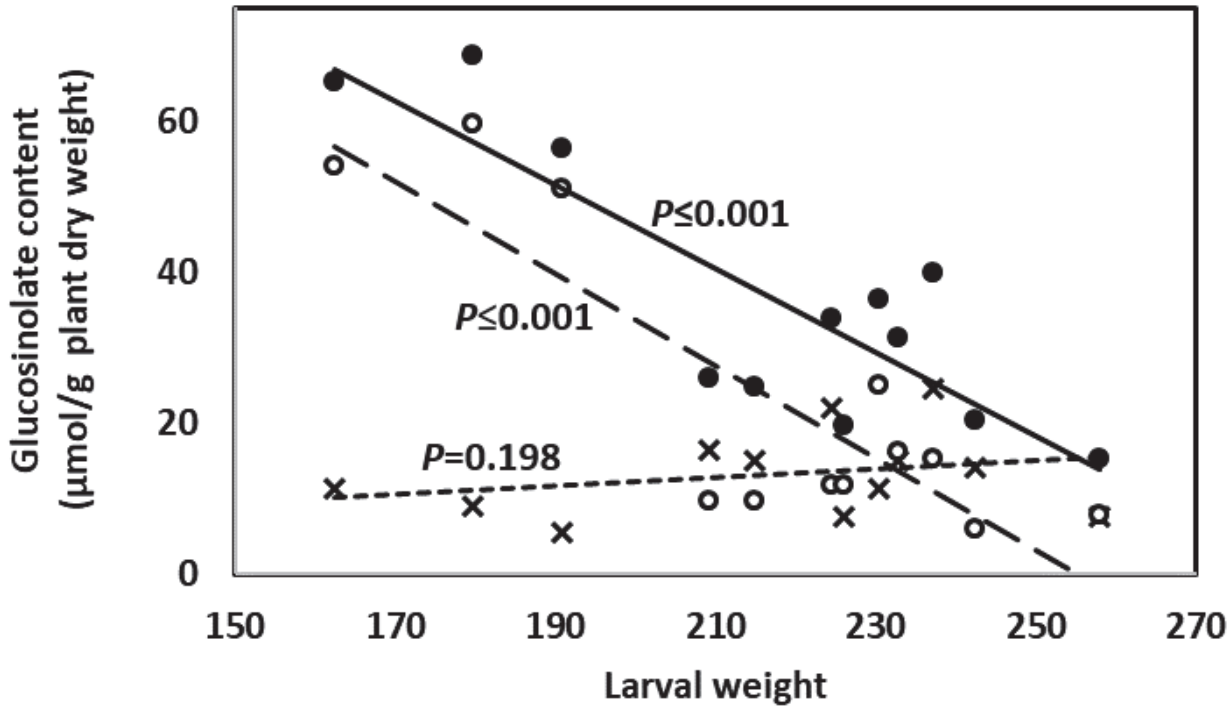


Figure 9. Mean ± SE larval weights after feeding on leaf discs of the different plant genotypes and treatments during 9 days (n = 8–10). Post hoc tests with a significance level of $p \leq 0.05$ were run to compare differences in larval weights within genotypes. Significant differences are shown with different lowercase letters.

In the HSIN genotype, the percentage of leaf discs with defoliation ≥50% was significantly lower in leaf discs from JA-treated plants than in leaf discs from control plants (Table S12). In the other treatments and genotypes, differences in percentage of leaf discs with defoliation ≥50% were not significantly different. According to this, after JA treatment of plants of the HSIN genotype, herbivory by *M. brassicae* larvae can be significantly reduced.

Weight gain in *M. brassicae* larvae and percentage of leaf discs with defoliation ≥50% were negatively correlated with IN content, the type most induced by JA application and by feeding of larvae of *M. brassicae*, and with TO content (Figures 10 and 11; Table S13). This was observed when considering glucosinolate content 3 and 9 days after treatment. Content of AL did not significantly affect weight gain in *M. brassicae* larvae (Table S13A), while the percentage of leaf discs with defoliation ≥50% was only affected by AL content when considering glucosinolate content at the end of the experiment (Table S13B).

(**A**)



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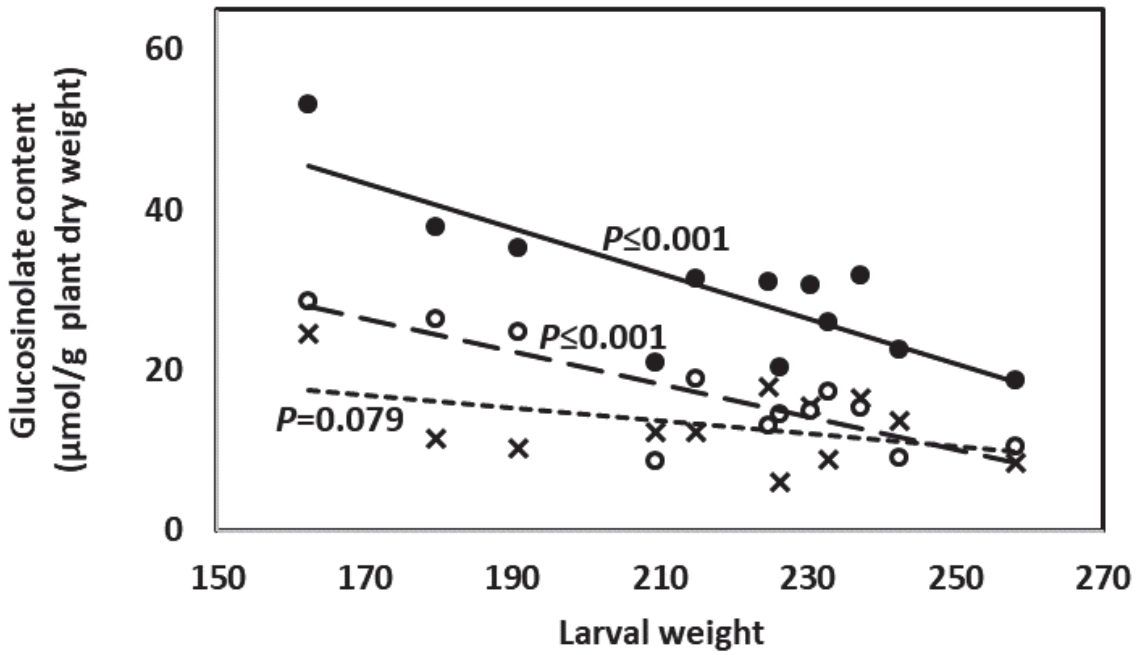

Figure 10. Pearson's correlations (significance level of $p \leq 0.05$) between plant glucosinolate content and larval weight at the end of the experiment considering glucosinolate content 3 days (**A**) and 9 days (**B**) after JA and SA treatment. Data used were the glucosinolate averages corresponding to each plant genotype (HGBS, LGBS, HSIN, and LSIN) and treatment (C, JA, and SA) (n = 12). Data points are crosses, white circles, and black circles for aliphatic, indolic, and total glucosinolates, respectively. Trends lines are short-dashed, long-dashed, and solid lines for aliphatic, indolic, and total glucosinolates, respectively. Significant differences are shown with different lowercase letters.

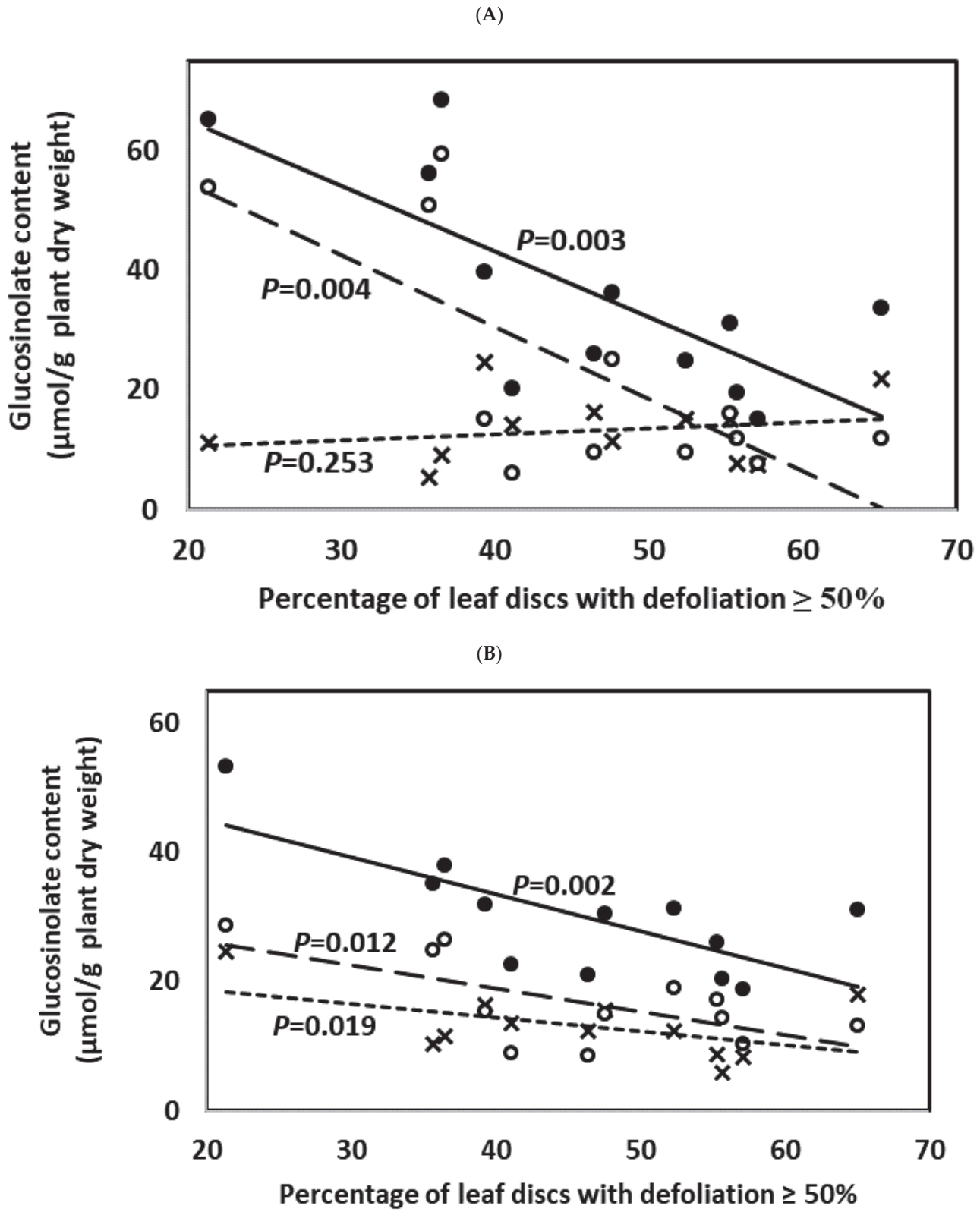

Figure 11. Pearson's correlations (significance level of $p \leq 0.05$) between plant glucosinolate content and herbivory (percentage of leaf discs with defoliation ≥50%) considering glucosinolate content 3 days (**A**) and 9 days (**B**) after JA and SA treatment. Data used were the glucosinolate averages corresponding to each plant genotype (HGBS, LGBS, HSIN, and LSIN) and treatment (C, JA, and SA) (n = 12). Data points are crosses, white circles, and black circles for aliphatic, indolic, and total glucosinolates, respectively. Trends lines are short-dashed, long-dashed, and solid lines for aliphatic, indolic, and total glucosinolates, respectively. Significant differences are shown with different lowercase letters.

3. Discussion

Previous studies have shown that there is an induction of glucosinolates as a result of herbivory, JA, and SA application [4,23,27]. Our study is the first to show differences in glucosinolate induction by herbivory and JA and SA application in different genotypes of the same crop through time and combining herbivory with JA and SA treatment. The four genotypes evaluated had been obtained through mass selection from the same variety, and thus they share the same genetic background with differences mostly due to glucosinolate composition [28]. Unlike our study with kale and *M. brassicae*, a previous study showed that low and high glucosinolate genotypes of *B. rapa* did not show changes in glucosinolate profiles as a result of feeding by the specialist root flies *Delia floralis* Fallén and *D. radicum* L. (Diptera: Anthomyiidae), despite differences in the expression of glucosinolate biosynthesis genes [29]. A different study also found differences in glucosinolate induction between wild and domesticated *B. oleracea* as a result of feeding by larvae of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) [10].

Our research shows that plant genotype affects glucosinolate induction in kale. In our experiments, application of JA and SA, and herbivory by *M. brassicae*, resulted in changes in foliar glucosinolate content that differed among plant genotypes. Application of JA application increased IN and TO contents at the three times tested in plants of the four plant genotypes tested, except in the LGBS genotype, which did not show any significant changes 9 days after JA treatment. The maximum IN and TO contents, which occurred at day 9 after the beginning of the experiment in control plants, were found to occur after 3 days in most of the genotypes in JA-treated and in JAL-treated plants. These findings show that both JA and herbivory induce a rapid accumulation of glucosinolates, particularly NEO and GBS in leaves. Similar results on genotype-specific induction of IN content were found either 2 or 4 days after methyl jasmonate treatment in *B. oleracea* and *B. rapa* [30,31]. Other studies have reported a maximum glucosinolate induction after JA and methyl jasmonate treatment in times varying from 1 to 7 days after treatment [32–34].

SA treatment was the most effective to induce content of AL, particularly GIB and SIN. However, induction of AL and TO was only noted for plants of the HGBS and LGBS genotypes 1 day after SA treatment, declining later. These findings agree with those found by a different study that found a significant increase in total glucosinolates in seedlings of *Brassica juncea* (L.) Czern. (Brassicaceae) 1 and 2 days after SA treatment [35]. Aliphatic glucosinolates have been reported to be more stable under different environmental conditions than indolic glucosinolates [36]. These findings were confirmed in our study. The variation in the contents of AL and the individual aliphatic glucosinolates SIN and GIB through time was less marked than the variation in the contents of IN content and the individual indolic glucosinolates GBS and NEO.

Our study also shows that as a result of larval feeding, differences in glucosinolate content among genotypes can change. For example, in the CL, JAL, and SAL treatments, IN contents in plants of the genotype HGBS were no longer higher than in genotype LGBS 3 and 9 days after phytohormone treatment and AL contents were no longer higher in plants of the HSIN genotype than in plants of the LSIN and HGBS genotypes 9 days after treatment.

The percent glucosinolate induction varied with plant genotype, the particular glucosinolate, and time after treatment. The most significant induction in IN content was noticed in the selection made by GBS and it was mostly due to NEO induction. NEO is known to be induced as a result of herbivory, and NEO reached its maximum percent increase 3 days after treatment in the HGBS genotype and 9 days after treatment in the LGBS genotype. IN showed a more significant induction compared to AL, thereby suggesting a prominent role of IN in plant defense response in kale. Similar results were reported previously from other crops like pak choi, *B. rapa* ssp. *chinensis* L., and Chinese cabbage, *B. rapa* ssp. *pekinensis* (Brassicaceae), in which increased accumulation of glucosinolates, particularly indolic ones like NEO, was observed 2–3 days after treatment with methyl jasmonate [34,37]. NEO is known to be induced as a result of herbivory by lepidopteran

larvae and plant pathogens [38,39]. Methoxylation modification of indolic glucosinolates is considered very important in plant defense against pathogens and this has been shown with 4-methoxyindol-3-ylmethyl (4-methoxyglucobrassicin, MEOHGBS) [40,41], which was a minor glucosinolate component in the genotypes included in this study. Our study shows that induction of NEO, 1-methoxyindol-3-ylmethyl, another methoxyindol glucosinolate, is very significant as a response to herbivory by *M. brassicae* larvae.

Despite differences in percent induction of glucosinolates among genotypes, there were no significant differences in GBS variation between plants of the HGBS and LGBS genotypes. In the case of SIN, there were only differences in SIN variation between the HSIN and LSIN genotypes in one case (in SA-treated plants 9 days after treatment). This indicates that there was limited trade-off between constitutive and induced GBS and SIN. A partial correlation between constitutive and induced glucosinolates, gene expression, and herbivory has also been found in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), suggesting that plant defense goes beyond individual metabolites or genes [42].

Many studies have found that glucosinolate content changes after herbivory in *Brassica* spp. and other plants [4,11,15]. Glucosinolate induction as a result of JA and SA application can also affect plant resistance [43]. Our research shows that plant genotype affects glucosinolate induction in kale after herbivory. In our study, in control plants with *M. brassicae* larvae (CL treatment), IN and TO contents tended to increase compared to control plants, but differences were not significant in all genotypes. The effect of herbivory alone (CL treatment) was not as strong on glucosinolate induction as the effect of JA treatment, indicating that the JA concentration we used was relatively high. We chose this concentration based on a study conducted with *A. thaliana* that used similar concentrations of JA and SA, producing significant induction of glucosinolates and reduction of larval growth in the generalist *Spodoptera exigua* [43]. The combined induction by both JA treatment and herbivory in the JAL treatment caused the largest induction in the glucosinolate NEO 3 days after treatment. However, AL content either was reduced or remained unaltered after herbivory, depending on the genotype. To our knowledge, no other studies have reported on a negative induction of AL content after feeding by generalist larvae. However, we only found a negative correlation between AL and IN content in one case, 3 days after treatment, in the CL treatment, while a positive correlation between AL and IN content was found 1 day after JA and SA treatment, 3 days after SA treatment, and 9 days after treatment in the SAL treatment. Overall, the interaction between previous application of SA and JA and feeding by larvae in the JAL and SAL treatments indicates that there was an enhancement or a cancellation of effects in JA- and SA-treated plants, respectively. Induction of indolic glucosinolates by *M. brassicae* and other generalist larvae has been found in other studies conducted with *A. thaliana* [24,25,44–46]. A lack of AL induction by generalist larvae has also been found in other studies conducted with *A. thaliana* [24,25,45–47], while a positive induction of AL in *tgg1 tgg2* mutant plants of *A. thaliana* that lack the major myrosinases was also reported by Badenes-Pérez et al. [24]. By contrast, feeding by larvae of the specialist *P. xylostella* resulted in negative induction of aliphatic and indolic glucosinolates in certain genotypes of *B. oleracea* and *A. thaliana*, respectively [10,24].

Our glucosinolate results refer particularly to the top two leaves of plants that were 6 weeks old at the beginning of the experiment, and through 9 days later. Over the 9-day length of the experiment, content of IN, TO, GBS, and NEO increased in control plants of the genotypes HGBS, LGBS, and HSIN, while in control plants of the genotype LSIN only SIN changed (increased). Plant age and leaf age within a plant are known to greatly affect glucosinolate content [48,49]. As glucosinolates can be induced as a result of herbivory, glucosinolate content is likely to have changed during the larval feeding and weight gain experiments compared to the glucosinolate data presented here for intact plants.

Mamestra brassicae and other generalist noctuids can be important pests in crops of the family Brassicaceae [50–53]. Larvae of *M. brassicae* have been found to cope well with some plant defense compounds, such as the triterpenoid saponins present in some

Barbarea spp. (Brassicaceae) that are resistant to other herbivores [54–57]. In the case of glucosinolates, aliphatic, indolic, and benzenic glucosinolates have been shown to have detrimental effects on *M. brassicae* larvae [9,11–13]. In this study, we found a negative correlation between larval weight gain in *M. brassicae* and IN and TO content, but not with AL content. This indicates that plant varieties with higher content of indolic glucosinolates, such as glucobrassicin, may be better equipped to defend themselves against herbivory by generalist herbivores like *M. brassicae*. Other studies with *M. brassicae* and other generalist herbivores, like *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), have found a negative correlation between aliphatic glucosinolate content and larval weight gain [9,11,58]. Besides glucosinolates, myrosinase activity can also cause resistance to *M. brassicae* [12,26]. However, using plant varieties with high glucosinolate content may increase susceptibility to specialist herbivores like *P. xylostella* [8]. For example, females of *P. xylostella* preferred ovipositing on *B. vulgaris* plants with a 20% higher content of the dominant glucosinolate glucobarbarin [59]. In this study, in the case of some treatments, like the JA and JAL treatments 3 days after treatment, we detected percentage increases in indolic glucosinolates that were much larger than 20%. Besides lepidopteran larvae, plant pathogens can also change glucosinolate content in Brassicaceae and changes can be variable depending on the plant species and cultivar [60–63]. For example, IN increased in plants of two *B. rapa* cultivars after infection with *Leptosphaeria maculans* and *Fusarium oxysporum*, but TO and AL increased in one cultivar and decreased in the other [60].

Application of methyl jasmonate also reduced herbivory by *M. brassicae* in ragwort, *Jacobaea* spp. (Asteraceae), and an increase in jacaranone, asparagine, threonine, isoleucine, and citric acid was associated to reduced herbivory after methyl jasmonate application [64]. Jacaranone has not been found in Brassicaceae, but some of these amino acids and citric acid are quite ubiquitously present in plants, so they could also be associated with reduced herbivory by *M. brassicae* in *B. oleracea* plants. Further research is necessary to study if other compounds besides glucosinolates might have played a role in the reduction of *M. brassicae* larval weights and herbivory that we detected in this study. In our study, reduced larval weights of *M. brassicae* were observed in JA-treated plants of the HGBS, HSIN, and LSIN genotypes, but a significant reduction of herbivory by *M. brassicae* larvae was only observed in JA-treated plants of the HSIN genotype. This indicates that, among the four genotypes that we tested, the HSIN genotype may be the best to conduct additional studies on the induction of plant defenses in kale.

4. Materials and Methods

4.1. Plants and Insects Used in the Experiments

Plants of kale, *B. oleracea* var. *acephala*, originated from a divergent selection program started in 2006 using kale population MBG-BRS0062 from the Brassica seedbank at Misión Biológica de Galicia (CSIC), northwestern Spain. The four kale genotypes tested had high (HSIN) and low sinigrin (LSIN) content and high (HGBS) and low glucobrassicin (LGBS) content [27]. Plants were grown in 18.7 cm diameter pots. Plants were 6 weeks old at the beginning of the experiments. *Mamestra brassicae* eggs were provided by the Centre de Recherches de Versailles (Versailles, France). After egg hatching, larvae were fed fresh cabbage leaves and were reared in plastic boxes in the laboratory (21 $\pm$ 3 °C, 65 $\pm$ 5 RH, and natural photoperiod).

4.2. Application of Phytohormones on Plants

A hand pump sprayer was used to apply approximately 14.3 mL/plant of either JA or SA in 0.5 mM concentrations including 0.1% Tween 20 (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany). Control plants were sprayed only with 0.1% Tween 20 solution. Treatment application on each individual plant lasted until runoff of the solution on plant foliage.

4.3. Interaction between Phytohormone and Herbivore Induction

One day after the application of phytohormones, 5 larvae per plant were placed on the leaves of 10 plants of each genotype and treatment. Thus, besides the control (C), JA, and SA treatments, three more treatments were included: control with larvae (CL), JA with larvae (JAL), and SA with larvae (SAL).

4.4. Analysis of Glucosinolates in Plants

To determine glucosinolate content, we harvested the top two leaves of each plant 1, 3, and 9 days after the application of phytohormones (3 and 9 days after the application of phytohormones in the case of the treatments with larvae, CL, JAL, and SAL). After freezing and freeze-drying these samples, glucosinolate content was analyzed as in Sotelo et al. [27]. Glucosinolate analysis was first used to determine the glucosinolate content in the control plants of each genotype. Thereafter, we focused on total aliphatic (AL), total indolic (IN), and total glucosinolate content (TO).

4.5. Herbivory and Larval Weight Gain Experiments

For each plant genotype and treatment, 10 third-instar larvae were individually placed in petri dishes of 9 cm diameter. Larvae were fed with leaf discs of 35 mm diameter collected from plants of the different treatments over a period of 9 days. Two middle leaves were used, i.e., any leaves except the top two ones and the bottom one. Larvae were inspected daily, and leaf discs were replaced by fresh ones on days 2 through 8. Feeding by larvae was visually assessed as having defoliated either $\geq$50% or <50% of the leaf disc. The total number of leaf discs with defoliation $\geq$50% were summed up at the end of the experiment, comparing the total to a potential maximum of one leaf disc for each of the 7 days in which herbivory was assessed. A percentage of leaf discs with defoliation $\geq$50% was calculated as the number of leaf discs with defoliation $\geq$50% divided by the potential maximum of one leaf disc per day multiplied by the number of replicates, which was 10 if none of the larvae had died from the beginning to the end of the experiment. The weights of larvae were measured at the beginning and end of the experiment (day 9) and the weight at day 9 was used to compare the effect of the different treatments and plant genotypes on larval weight gain.

4.6. Statistical Analyses

Differences in glucosinolate content among plants of each genotype, phytohormone and herbivory treatment, and time (days after phytohormone treatment) were analyzed using either one-way ANOVA, if data were parametric, followed by either LSD or Tamhane, or Kruskal–Wallis and Mann–Whitney tests ($p \leq 0.05$) using SPSS® version 26 (IBM Corp., Armonk, NY, USA). In the post hoc analysis after the Kruskal–Wallis tests, the *p*-value that we used was the one with the adjusted significance, adjusted by the Bonferroni correction for multiple tests. Percentages of leaf discs consumed over a period of 9 days, were analyzed using a one-tailed, two-sample test of proportions using STATA® version 15.1 with significance at $p \leq 0.05$. Differences in larval weights among the different treatments and species were analyzed using Kruskal–Wallis tests, using the significance adjusted by the Bonferroni correction in post hoc analysis. Correlations between aliphatic and indolic glucosinolate induction were performed using one-tailed Spearman's rho correlations with SPSS®. Correlations between leaf disc consumption and larval weights with glucosinolate content were performed using one-tailed Pearson correlations with SPSS®.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10091951/s1, Table S1: Glucosinolate content for each time (days after treatment), genotype, and treatment (n = 7–10). The treatments are control (C), jasmonic acid (JA), salicylic acid (SA), control with *M. brassicae* larvae (CL), JA with M. brassicae larvae (JAL), and SA with *M. brassicae* larvae (SAL). The genotypes are high in glucobrassicin (HGBS), low in glucobrassicin

(LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). The glucosinolates shown are progoitrin (PRO), glucoiberverin (GIV), 4-hydroxyglucobrassicin (OHGBS), 4-methoxyglucobrassicin (MEOHGBS), neoglucobrassicin (NEO), and gluconasturtiin (GNT). Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively; Table S2: Differences in glucosinolate content across times (days after treatment with JA and SA). Test statistic and p-values of ANOVA or Kruskall–Wallis test shown to compare differences in glucosinolate content among times (1, 3, and 9 days after treatment in the case of the treatments C, JA, and SA, and 3 and 9 days after treatment in the case of CL, JAL, and SAL) within the same genotype. Significant p-values ($p \leq 0.05$) are shown in bold type; Table S3: Mean ± SE glucosinolate content (μmol g^{-1} plant dry weight) for each treatment and genotype after the application of phytohormones, 1, 3, and 9 days after treatment. The treatments are control (C), jasmonic acid (JA), salicylic acid (SA), control with M. brassicae larvae (CL), JA with M. brassicae larvae (JAL), and SA with M. brassicae larvae (SAL). The genotypes are high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). The glucosinolates shown are glucoiberin (GIB), sinigrin (SIN), glucobrassicin (GBS), neoglucobrassicin (NEO), total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). The less abundant glucosinolates progoitrin (PRO), glucoiberverin (GIV), 4-hydroxyglucobrassicin (OHGBS), 4-methoxyglucobrassicin (MEOHGBS), and gluconasturtiin (GNT) are not shown here, but are shown as supplementary data. For each genotype and treatment, means within a column followed by different letters show significant differences ($p \leq 0.05$) in time (days after treatment). Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively; Table S4: Effect of JA, SA, CL, JAL, and SAL treatments on glucosinolate content in the different genotypes, 1, 3, and 9 days after treatment with JA and SA. Test statistic and p-values of ANOVA or Kruskall–Wallis test shown to compare differences in glucosinolate content among treatments within the same genotype. Treatments included in the comparisons among treatments are C, JA, and SA (also CL for 3 and 9 days after treatment) (A), and JA compared to JAL and SA compared to SAL (3 and 9 days after treatment) (B). Significant p-values ($p \leq 0.05$) are shown in bold type; Table S5: Mean ± SE glucosinolate content (μmol g^{-1} plant dry weight) for each treatment and genotype after the application of phytohormones. The treatments are control (C), jasmonic acid (JA), salicylic acid (SA), and control with *M. brassicae* larvae (CL). The genotypes are high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN). The glucosinolates shown are glucoiberin (GIB), sinigrin (SIN), glucobrassicin (GBS), neoglucobrassicin (NEO), total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). The less abundant glucosinolates progoitrin (PRO), glucoiberverin (GIV), 4-hydroxyglucobrassicin (OHGBS), 4-methoxyglucobrassicin (MEOHGBS), and gluconasturtiin (GNT) are not shown here, but are shown as supplementary data. For each time (days after treatment) and genotype, means within a column followed by different letters show significant treatment differences ($p \leq 0.05$) among genotypes. Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively; Table S6: Mean ± SE glucosinolate content (μmol g^{-1} plant dry weight) in kale genotypes after the application of phytohormones to genotypes high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin (HSIN), and low in sinigrin (LSIN) (n = 3–10). The treatments are jasmonic acid (JA), salicylic acid (SA), JA with M. brassicae larvae (JAL), and SA with M. brassicae larvae (SAL). The glucosinolates shown glucoiberin (GIB), sinigrin (SIN), glucobrassicin (GBS), neoglucobrassicin (NEO), total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). The less abundant glucosinolates progoitrin (PRO), glucoiberverin (GIV), 4-hydroxyglucobrassicin (OHGBS), 4-methoxyglucobrassicin (MEOHGBS), and gluconasturtiin (GNT) are shown as supplementary data. For each time (days after treatment) and genotype, means within a column followed by different letters show significant treatment differences ($p \leq 0.05$) among genotypes. Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively; Table S7: Changes in glucosinolate content among genotypes under C, JA, SA, CL, JAL treatments 1, 3, and 9 days after treatment with JA and SA. Test statistic and p-values of ANOVA or Kruskall–Wallis test shown to compare differences in glucosinolate content among genotypes subject to the same treatment. Significant p-values ($p \leq 0.05$) are shown in bold type; Table S8: Mean ± SE glucosinolate content (μmol g^{-1} plant dry weight) for each treatment and genotype after the application of phytohormones (n = 3–10). The treatments are control (C), jasmonic acid (JA), salicylic acid (SA), control with M. brassicae larvae (CL), JA with M. brassicae larvae (JAL), and SA with M. brassicae larvae (SAL). The genotypes are high in glucobrassicin (HGBS), low in glucobrassicin (LGBS), high in sinigrin

(HSIN), and low in sinigrin (LSIN). The glucosinolates shown are glucoiberin (GIB), sinigrin (SIN), glucobrassicin (GBS), neoglucobrassicin (NEO), total aliphatic (AL), total indolic (IN), and total glucosinolates (TO). The less abundant glucosinolates progoitrin (PRO), glucoiberverin (GIV), 4-hydroxyglucobrassicin (OHGBS), 4-methoxyglucobrassicin (MEOHGBS), and gluconasturtiin (GNT) are not shown here, but are shown as supplementary data. For each time (days after treatment) and treatment, means within a column followed by different letters show significant differences
($p \leq 0.05$) among genotypes. Replication was n = 7–10, n = 5–10, and n = 3–5 for 1, 3, and 9 days after treatment, respectively; Table S9: Effect of JA, SA, CL, JAL, and SAL treatments on glucosinolate content in the different genotypes, 1, 3, and 9 days after treatment with JA and SA. Test statistic and p-values of ANOVA or Kruskall–Wallis test shown to compare differences in glucosinolate content (percentages, compared to the control within each genotype) among genotypes subject to the same treatment. Significant p-values ($p \leq 0.05$) are shown in bold type; Table S10: Significance of correlations between aliphatic (AL) and indolic (IN) glucosinolate in induced plants. Data used in the correlations included all glucosinolate data from the four plant genotypes (HGBS, LGBS, HSIN, and LSIN) for each of the treatments, 1, 3 and 9 days after JA and SA treatment (n = 39–40) and 3 and 9 days after CL, JAL, and SAL treatments began (n = 19–20). Significant p-values ($p \leq 0.05$) of one-tailed Spearman's rho correlation are shown in bold type; Table S11: Differences in larval weights after feeding on leaf discs of the different plant genotypes and treatments during 9 days (n = 8–10). p-values from Mann-Whitney U tests. Significant p-values ($p \leq 0.05$) are shown in bold type; Table S12: Comparison of the percentage of leaf discs with defoliation $\geq$50% as a result of larval feeding under control (C), jasmonic acid (JA), and salicylic acid (SA) treatments. Significant p-values ($p \leq 0.05$) of one-tailed two-sample tests of proportions are shown in bold type; and Table S13. Significance of correlations between plant glucosinolate content and larval weight at the end of the experiment (A) and between plant glucosinolate content and percentage of leaf discs with defoliation $\geq$ 50% (B). Correlations are shown for glucosinolate content 3 days and 9 days after JA and SA treatment. Data used were the glucosinolate averages corresponding to each plant genotype (HGBS, LGBS, HSIN, and LSIN) and treatment (C, JA, and SA) (n = 12). Three different classes of glucosinolates were distinguished, aliphatic (AL), indolic (IN), and total (TO). Significant p-values ($p \leq 0.05$) are shown in bold type.

Author Contributions: F.R.B.-P. and M.E.C. conceived and designed the research; M.E.C. and F.R.B.-P. conducted the bioassay experiments; M.E.C. analyzed the glucosinolate content of plants; F.R.B.-P. and M.E.C. analyzed the data; and F.R.B.-P. wrote the paper; M.E.C. provided comments and additions. Both authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Science, Innovation, and Universities, grant RTI2018-096591-B-I00.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank Pablo Velasco for help with glucosinolate analysis and Rosaura Abilleira and Juan Carlos Hernández for technical help with the experiments.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Morphological and Chemical Factors Related to Western Flower Thrips Resistance in the Ornamental Gladiolus

Dinar S. C. Wahyuni [1,2], Young Hae Choi [3,4], Kirsten A. Leiss [5] and Peter G. L. Klinkhamer [1,*]

1 Plant Science and Natural Products, Institute of Biology (IBL), Leiden University, Sylviusweg 72, 2333BE Leiden, The Netherlands; dinarsari_cw@staff.uns.ac.id
2 Pharmacy Department, Faculty Mathematics and Natural Sciences, Universitas Sebelas Maret, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
3 Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333BE Leiden, The Netherlands; y.choi@chem.leidenuniv.nl
4 College of Pharmacy, Kyung Hee University, Seoul 02447, Korea
5 Business Unit Horticulture, Wageningen University and Research Center, Postbus 20, 2665ZG Bleiswijk, The Netherlands; kirsten.leiss@wur.nl
* Correspondence: p.g.l.klinkhamer@biology.leidenuniv.nl; Tel.: +31-71-527-5158

Abstract: Understanding the mechanisms involved in host plant resistance opens the way for improved resistance breeding programs by using the traits involved as markers. Pest management is a major problem in cultivation of ornamentals. Gladiolus (*Gladiolus hybridus* L.) is an economically important ornamental in the Netherlands. Gladiolus is especially sensitive to attack by western flower thrips (*Frankliniella occidentalis* (Pergande) (Thysanoptera:Thripidae)). The objective of this study was, therefore, to investigate morphological and chemical markers for resistance breeding to western flower thrips in Gladiolus varieties. We measured thrips damage of 14 Gladiolus varieties in a whole-plant thrips bioassay and related this to morphological traits with a focus on papillae density. Moreover, we studied chemical host plant resistance to using an eco-metabolomic approach comparing the ^{1}H NMR profiles of thrips resistant and susceptible varieties representing a broad range of papillae densities. Thrips damage varied strongly among varieties: the most susceptible variety showed 130 times more damage than the most resistant one. Varieties with low thrips damage had shorter mesophylls and epidermal cells, as well as a higher density of epicuticular papillae. All three traits related to thrips damage were highly correlated with each other. We observed a number of metabolites related to resistance against thrips: two unidentified triterpenoid saponins and the amino acids alanine and threonine. All these compounds were highly correlated amongst each other as well as to the density of papillae. These correlations suggest that papillae are involved in resistance to thrips by producing and/or storing compounds causing thrips resistance. Although it is not possible to distinguish the individual effects of morphological and chemical traits statistically, our results show that papillae density is an easy marker in Gladiolus-breeding programs targeted at increased resistance to thrips.

Keywords: Gladiolus; *Frankliniella occidentalis*; host plant resistance; morphological markers; mesophyll; epidermis; papillae; eco-metabolomics

1. Introduction

Sustainable growth and development require minimizing the natural resources and toxic materials used, and the waste and pollutants generated, throughout the entire production and consumption process. This also applies to the production of food and ornamentals, the sustainable production of which requires minimizing the use of pesticides. Breeding for resistance becomes more and more important in this respect. Nowadays, we can apply molecular tools, such as gene expression and the use of mutants, to many species to discover mechanisms of host plant resistance. Such methods and techniques

have become increasingly cheaper and, at some point, will become available for all crops. However, the need to reduce pesticides is extremely urgent as was recently again signaled by reports on the alarming decline in insect species [1]. For some crops, especially polyploids and crops with large genomes, feasible, required molecular tools will most likely not become available soon. So, the market still calls for fast and cheap alternatives such as morphological or chemical markers.

Plant defense against insect herbivores comprises morphological traits, such as spines, trichomes, and papillae, as well as chemical traits. Trichomes are epidermal hairs protruding from the surface of leaves and stems [2], impeding movement or trapping herbivorous insects, resulting in their death [3–5]. Papillae are protuberances of solid cell wall thickening [3]. They are known to protect cells from pathogen attack due to physical barriers [4,5]. Prum et al. [6] showed that papillae made it more difficult for beetles to attach to the leaves, suggesting that papillae also play a role in defense against insect herbivores. Despite this potential role, papillae have not been studied in great detail yet in relation to insect plant defense.

Besides physical barriers, both trichomes and papillae can produce secondary metabolites that deter or are toxic to herbivores. Among those secondary metabolites are glucosinolates, alkaloids, phenolics, phenylpropanoids, polyketides, and terpenoids [7]. For instance, acylsugars [8] and phenols [9] are produced in trichomes of *Solanum pennellii*, while phenolics and alkaloids were detected in trichomes of *Withania somnifera* L. [10]. Papillae store plant defense compounds such as 2-acetyl-1-pyrroline in rice [11] and cardosin A in *Cynara cardunculus* L. [12].

Because trichomes and papillae are part of the plant's defense system, they may play an important role in breeding programs aimed at increasing natural host plant resistance. Host plant resistance becomes increasingly important in integrated pest management programs directed at agricultural and horticultural key pests such as western flower thrips (*Frankliniella occidentalis*). This invasive pest is highly polyphagous and attacks fruits, vegetables, and ornamentals [13,14]. It has been recorded feeding on over 250 crop species from over 60 families (15), causing losses of millions of euros worldwide [14–16]. Thrips have piercing–sucking mouthparts allowing them to suck up a whole cell's contents, leaving an empty cell filled with air, causing a characteristic silver leaf scar, the so-called silver damage [17]. Adult thrips and larvae feed in the same manner, both contributing to the damage [14]. Females feed more intensively than males, which is attributed to their lower mobility and high consumption rates needed for egg production [18]. Feeding on actively growing tissues leads to stunting and distorted plant growth with eventual yield loss [19]. In addition, feeding injury causes a reduction in the aesthetic value and storage quality of the produce [20]. In addition, thrips is an important vector of viral diseases [21].

Host plant resistance to western flower thrips is a promising approach hampering preference, reproduction, feeding, and/or transmission of virus [22]. It is mainly chemically based in a number of plants species, as shown by Leiss et al. [23] applying an eco-metabolomic approach. They compared the ^{1}H nuclear magnetic resonance (NMR) spectroscopy profiles of thrips-resistant and thrips-susceptible plants to identify metabolites related to constitutive thrips resistance in wild *Jacobaea* species [24], in the ornamental chrysanthemum [25], and in the vegetables tomato [9,26] and carrot [27], which showed alkaloids, acylsugars, flavonoids, amino acids, and phenylpropanoids, respectively. In addition, eco-metabolomics has been applied to determine metabolites related to thrips resistance in sweet pepper [28,29] and onion [30], which showed triterpenoid derivatives.

Gladiolus, a genus of perennial bulbs, belongs to the Iridaceae family. It is a popular decorative plant in summer and thus constitutes an economically important flower crop in the Netherlands. *Gladiolus* comprises 5% of the total Dutch flower production, constituting 21,000 ha of the production area and amounting to $756 million production in value [31]. Western flower thrips is a major problem in the cultivation of Gladiolus, affecting corms, leaves, buds, and flowers. Thrips damage results in small corms, which

may not germinate; problems of flower formation and opening; and an undesirable silvery, shiny damage on leaves and flowers [19].

The aim of this study was to identify morphological and chemical markers for resistance to western flower thrips, looking at feeding damage, that can be used in breeding programs of Gladiolus. In particular, we wanted to address the following questions: (1) Does thrips damage vary among Gladiolus varieties? (2) Is thrips damage related to morphological traits? (3) Is thrips resistance based on chemical traits, and if so, which compounds are involved? (4) Are the morphological and chemical traits mutually correlated? (5) Which (combinations of) traits provide the best marker for thrips resistance in breeding programs? We focused on the following morphological traits: plant dry mass, leaf length, size of epidermal cells, size of mesophylls, and density of papillae at the leaf surface. After we established that thrips resistance is related to several morphological traits, we continued using leaf extracts to show that resistance was at least partly based on plant chemistry. We then compared the ^{1}H NMR profiles and thrips resistance of gladiolus varieties representing a broad range of papillae densities to identify potential metabolites related to resistance.

2. Results

2.1. Differences in Resistance to Thrips

Thrips silver damage in the whole-plant bioassay differed significantly among varieties (F = 11.445, df = 13, p = 0.000). Charming Beauty and Charming, as the most susceptible varieties, showed significantly more damage compared with all other varieties, while Robinetta and Alba showed almost no damage at all (Figure 1). Charming displaying the highest amount of damage (mean 3159.3 $\pm$ 434.8 mm^2), showed 130 times more damage than Robinetta (mean 23.8 $\pm$ 8.9 mm^2).

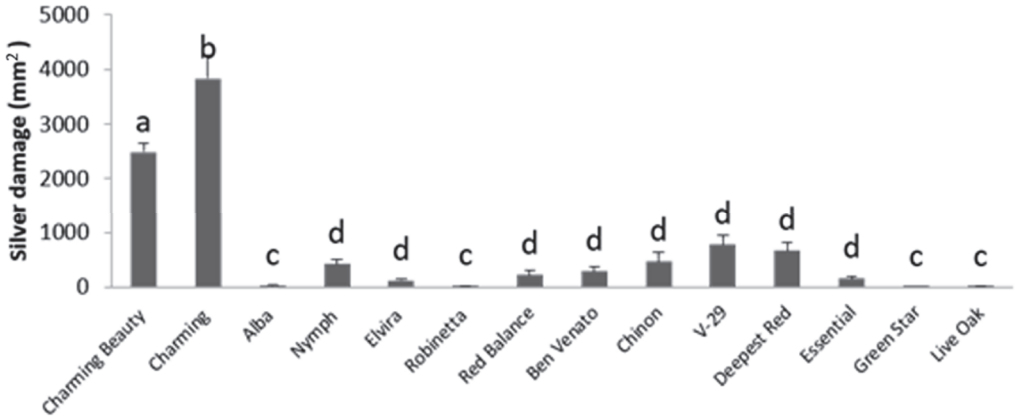

Figure 1. Silver damage (mm^2) in 14 Gladiolus varieties, as measured by a whole-plant thrips non-choice bioassay. Data represent means and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.

2.1.1. Differences in Morphological Traits

Leaf length (F = 15.522, df = 13, p = 0.000) (Figure 2A), the length of epidermal cells (F = 125.459, df = 13, p = 0.000) (Figure 3A) and mesophylls (F = 90.136, df = 13, p = 0.000) (Figure 3B), and the density of epicuticular papillae (F = 29.363, df = 13, p = 0.000) all differed significantly between varieties. These morphological characteristics were mutually highly correlated. This can be explained by the fact that as a rule, each epidermal cell produces one papilla (Figure 4E,F). The different leaf cell lengths as well as papillae density of the thrips-susceptible variety Charming Beauty compared with the thrips-resistant variety Robinetta are depicted as microscopy images in Figure 4A–F. In general, susceptible varieties had longer leaves and cells and lower densities of papillae.

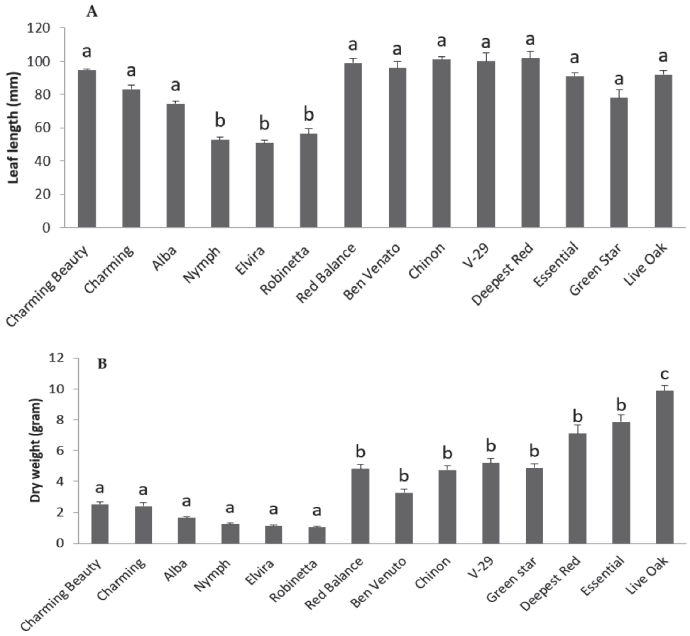

Figure 2. Leaf length (**A**) and dry mass (**B**) of 14 Gladiolus varieties. Data represent means and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.

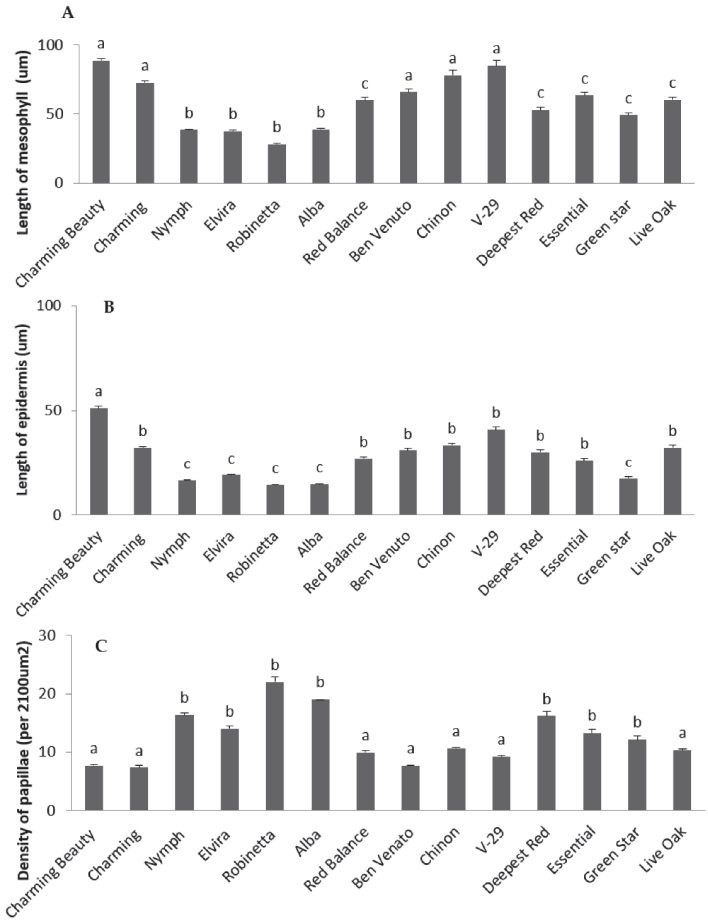

Figure 3. The length of mesophylls (**A**) and epidermal cells (**B**) and the density of papillae (**C**) in 14 Gladiolus varieties. Data represent means and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.

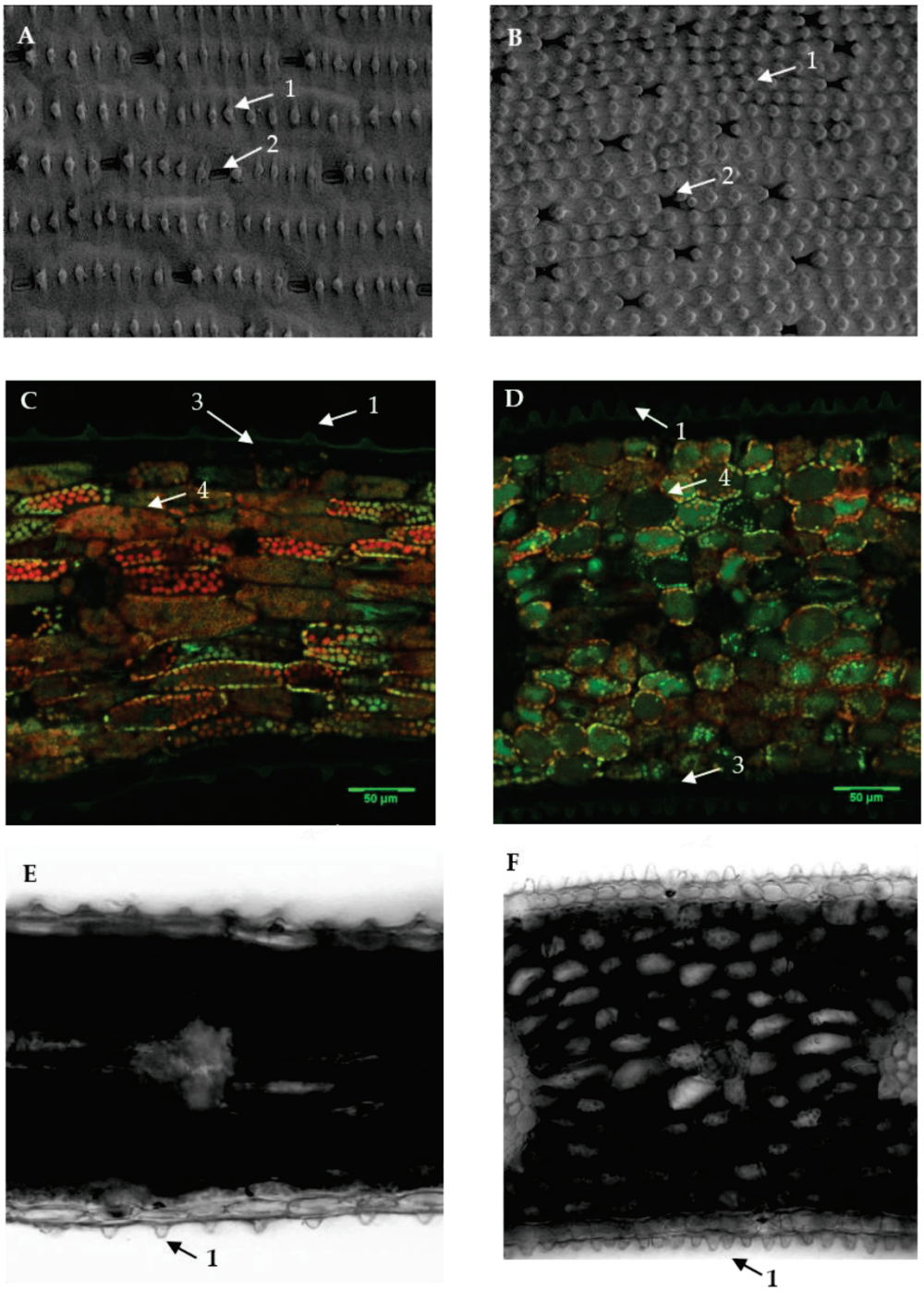

Figure 4. Leaf surface scanning electron photomicrographs of the thrips-susceptible Gladiolus variety Charming Beauty (**A**) and the thrips-resistant variety Robinetta (**B**). Lead cross sections of Charming Beauty (**C**) and Robinetta (**D**) with confocal laser scanning. Leaf cross sections of Charming Beauty (**E**) and Robinetta (**F**) with visual light microscopy. Arrows indicate papillae (1), stomata (2), epidermal cells, (3) and mesophyll (4).

In addition, the dry mass of varieties differed significantly (F = 70.531, df = 13, $p = 0.000$), with large size varieties yielding more than double the dry mass compared with the small ones (Figure 2B). Dry mass was not significantly correlated with the other morphological characteristics.

2.1.2. The Relationship between Thrips Damage and Morphological Characteristics

Silver damage was significantly positive correlated with the length of the epidermal cells (r = 0.596, N = 14, p = 0.024) (Figure 5A) and mesophylls (r = 0.603, N = 14, p = 0.022) (Figure 5B), while it was significantly negatively correlated with the density of papillae (r = -0.628, N = 14, p = 0.016) (Figure 5C). Silver damage did not correlate with leaf length (r = 0.320, N = 14, p = 0.264) or plant dry mass (r = −0.222, N = 14, p = 0.445).

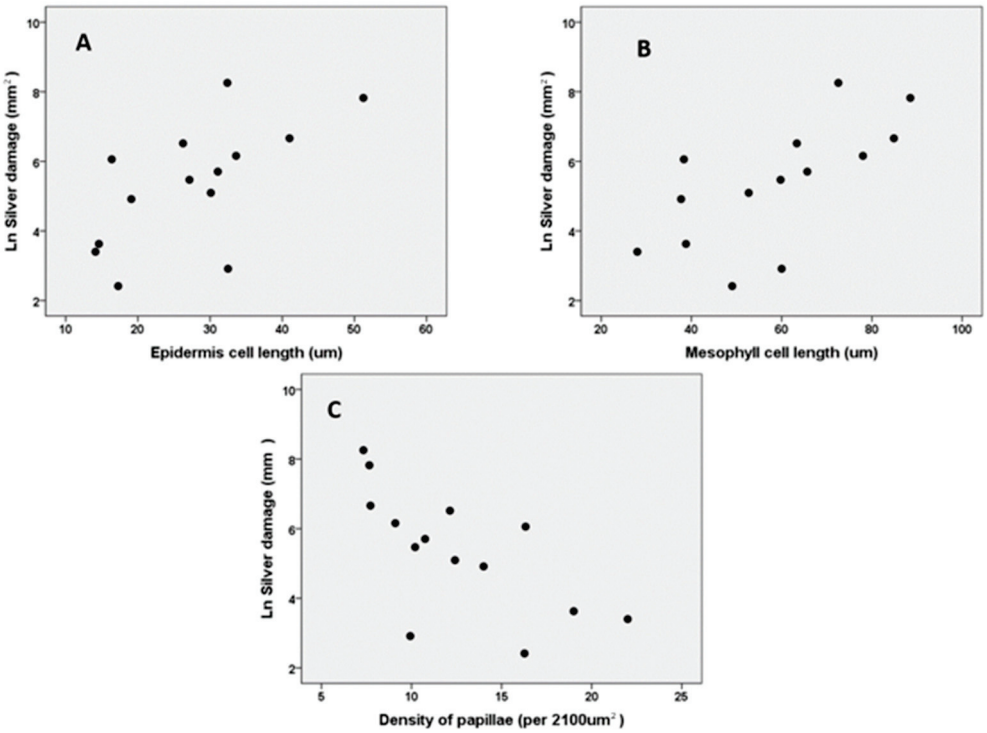

Figure 5. Correlation between thrips silver damage, measured in a whole-plant non-choice bioassay, and cell length of epidermal cells (**A**) (r = 0.596, N = 14, p = 0.024) and mesophylls (**B**) (r = 0.603, N = 14, p = 0.022) and density of epidermal papillae (**C**) (r = −0.628, N = 14, p = 0.016) in 14 Gladiolus varieties.

2.2. Chemical Study

2.2.1. Differences in the Effect of Six Leaf Extracts on Thrips Mortality

To show that thrips resistance in Gladiolus is at least partly based on chemical characteristics, we studied the effects of leaf extracts of six varieties in artificial diets on thrips mortality. The extracts of the following four varieties with low thrips damage in the previously described whole-plant non-choice bioassay lead to significantly higher thrips mortality compared to the negative control (Figure 6A): Alba (χ^2= 7.59, d.f. = 1, p = 0.005) Nymph (χ^2= 10.26, d.f. = 1, p = 0.001), Elvira (χ^2= 13.17, d.f. = 1, p = 0.0003), and Robinetta, (χ^2= 8.89, d.f. = 1, p = 0.002). The extracts of two varieties with high silver damage, Charming Beauty and Charming, showed a thrips mortality comparable to the negative control.

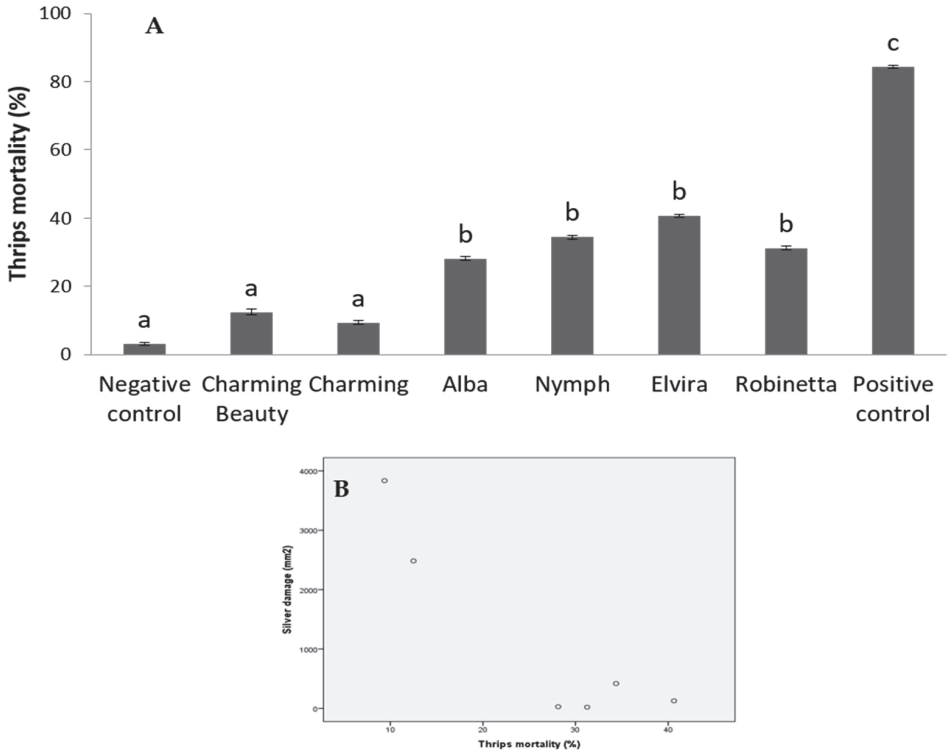

Figure 6. (**A**) Mortality of thrips feeding on artificial diets (150 µL of 2% agarose) with 50 µL of leaf extracts of six *Gladiolus* varieties measured in an in vitro bioassay. For each extract, 32 thrips were tested 5% methanol solution was used as a negative control and the insecticide abamectin (50 µg/mL) as a positive control. Means and standard errors are presented. Different letters indicate significant differences between varieties at $p \leq 0.05$. (**B**) Correlation between thrips silver damage, measured in a whole-plant non-choice bioassay, and thrips mortality measured in the in vitro bioassay of six *Gladiolus* varieties ($r = -0.788$, $N = 6$, $p = 0.031$).

Thrips mortality in this in vitro bioassay was negatively correlated with thrips silver damage in the whole-plant non-choice bioassay ($r = -0.788$, $N = 6$, $p = 0.031$) (Figure 6B). This result implied that chemical compounds play a role in plants' resistance to thrips. We, therefore, continued with the chemical profiling of all varieties.

2.2.2. Metabolic Profiling

In addition to variation in thrips resistance, the Gladiolus varieties differed in their metabolomic profiles. PCA, which is an unsupervised method, did not give a separation of metabolic profiles based on thrips resistance of the varieties (Figure S1). The supervised orthogonal partial least squares (OPLS)–discriminant analysis (DA), in contrast to PCA, takes into account, in addition to the metabolomic matrix, the resistance matrix. We, therefore, separated varieties into resistant (0.03–0.8% damage), partially resistant (4–20% damage), and susceptible (>65% damage) varieties. The loading plot identified candidate signals related to thrips resistance, as shown in the positive value, and candidate signals related to susceptibility, as shown in the negative value (Figure 7). Validation of OPLS-DA by permutation tests resulted in variance $R^2 = 0.93$ and predictive ability $Q^2 = 0.88$. Q^2 values greater than 0.5 are generally accepted as good [32].

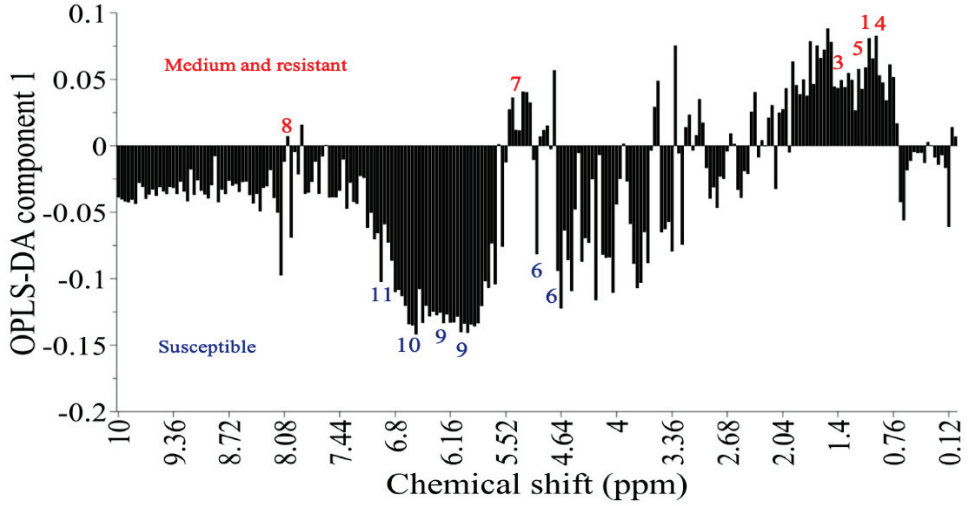

Figure 7. Loading plot for OPLS-DA of Gladiolus varieties based on ^{1}H NMR spectra. Metabolites are labeled as (1) signal A, (2) signal B, (3) alanine, (4) valine, (5) threonine, (6) glucose, (7) sucrose, (8) kaempferol, (9) epicatechin, (10) epigallocatechin, and (11) gallic acid.

2.2.3. Signals Related to Resistance

The signals related to thrips resistance were observed in the region of 0.80–1.92 ppm (Figure 7). Compounds showing signals in this region are terpenoids, saponins, and amino acids. Signal A (δ 1.28) and signal B (δ 0.90) were associated with thrips-resistant varieties and belonged to the triterpenoid saponins. However, these remained unidentified due to overlapping signals in ^{1}H NMR spectra. We further identified signals related to several amino acids: valine (δ 1.06), alanine (δ 1.48), and threonine (δ 1.32). In addition, the signal of sucrose (δ 5.40) was also related to thrips resistance.

The relative concentrations of alanine, valine, and threonine differed significantly among varieties (F = 21.754, df = 13, p = 0.000; F = 75.824, df = 13, p = 0.000; and F = 31.460, df = 13, p = 0.000). The relative concentrations of alanine, valine, and threonine were three to four times higher in resistant varieties (Table 1). The relative concentrations of alanine and threonine were negatively correlated to thrips silver damage (r = −0.612, N = 14, p = 0.020 and r = −0.634, N = 14, p = 0.015, respectively), while the relative concentration of valine was not significantly correlated to thrips silver damage (r = −0.100, N = 14, p = 0.734).

The relative concentrations of signals A and B were significantly different among varieties (F = 52.216, df = 13, p = 0.000 and F = 44.563, df = 13, p = 0.000). Signals A (r = −0.505, N = 14, p = 0.065) and B (r = −0.557, N = 14, p = 0.038) (Table 2) were negatively correlated with silver damage, although for signal A, this was only marginally significant (Table 2). The relative concentration of sucrose differed among varieties (F = 14.367, df = 13, p = 0.000). Although the concentration of sucrose was about 1.15 times higher in resistant varieties than in susceptible varieties, we did not detect a significant correlation between silver damage and sucrose concentration (r = 0.083, N = 14, p = 0.779) (Table S1). In conclusion, three of the compounds related to resistance were confirmed to be important in subsequent analyses of relative concentrations of the single compounds in univariate correlations. These compounds were the amino acids alanine and threonine and the compound related to signal B (δ 0.90).

Table 1. Pearson correlations (N = 14) between ln-thrips silver damage (mm^2) and epidermal cell length (μm), mesophyll length (μm), density of papillae (per 2100 μm^2), leaf length (cm), and dry mass (g) in Gladiolus varieties (N = 14). Data represent means of three to five replicates. Data in bold show the significance level at $p \leq 0.05$.

	Epidermal Cell Length (μm)	**Mesophyll Length (μm)**	**Density of Papillae (per 2100 μm^2)**	**Leaf Length (cm)**	**Dry Mass (g)**
Ln silver damage	$r = 0.596$ $p = 0.024$	$r = 0.603$ $p = 0.022$	$r = -0.628$ $p = 0.016$	$r = 0.320$ $p = 0.264$	$r = -0.222$ $p = 0.445$
Epidermal cell length		$r = 0.931$ $p = 0.000$	$r = -0.873$ $p = 0.000$	$r = 0.704$ $p = 0.005$	$r = 0.310$ $p = 0.281$
Mesophyll length			$r = -0.909$ $p = 0.000$	$r = 0.777$ $p = 0.001$	$r = 0.315$ $p = 0.273$
Density of papillae				$r = -0.669$ $p = 0.009$	$r = -0.389$ $p = 0.170$
Leaf length					$r = 0.441$ $p = 0.114$

Table 2. Correlations between the concentrations of metabolites that are not related to thrips resistance and silver damage and the density of papillae in 14 Gladiolus varieties. Silver damage was ln-transformed to obtain normally distributed data. * = $p < 0.05$ and N = 14 in all cases. Spearman correlations were used for the relationships between silver damage, epigallocatechin (EGC), gallic acid, and density of papillae.

	Valine	**Sucrose**	**Glucose**	**EGC**	**Gallic Acid**
Ln damage	$r = -0.100$ $p = 0.734$	$r = 0.083$ $p = 0.779$	$r = 0.265$ $p = 0.360$	$r = 0.404$ $p = 0.152$	$r = 0.313$ $p = 0.276$
Papillae	$r = -0.034$ $p = 0.907$	$r = -0.117$ $p = 0.692$	$r = -0.442$ $p = 0.114$	$r = -0.275$ $p = 0.342$	$r = -0.019$ $p = 0.950$
Valine		$r = 0.345$ $p = 0.227$	$r = 0.096$ $p = 0.743$	$r = -0.594$ * $p = 0.025$	$r = -0.403$ $p = 0.153$
Sucrose			$r = -0.123$ $p = 0.676$	$r = -0.576$ * $p = 0.031$	$r = -0.058$ $p = 0.845$
Glucose				$r = -0.047$ $p = 0.874$	$r = 0.139$ $p = 0.636$
EGC					$r = 0.074$ $p = 0.801$

2.2.4. Signals Related to Susceptibility

Glucose was related to susceptibility (Figure 7) and differed significantly (F = 8.352, df = 13, $p < 0.000$ and F = 8.234, df = 13, $p < 0.000$) among varieties (Table S1). These signals were, however, not correlated to thrips silver damage (r = 0.234, N = 14, p = 0.420 and r = 0.265, N = 14, p = 0.360) when tested as a single factor (Table 2). Epicatechin, epigallocatechin, and gallic acid were also related to susceptibility. These signals were not detectable in all varieties. The relative concentrations of epicatechin, epigallocatechin, and gallic acid differed significantly among varieties (X^2 = 52.132, df = 13, p = 0.000; $-X^2$ = 49.133, df = 13, p = 0.000; and X^2 = 48.397, df = 13, p = 0.000, respectively). Epicatechin was marginally significantly related to thrips resistance (ρ = 0.541, N = 14, p = 0.046); it was present, however, in only three varieties. Epigallocatechin and gallic acid were not related to thrips silver damage when tested as a single factor (ρ = 0.404, N = 14, p = 0.152 and ρ = 0.313, N = 14, p = 0.276, respectively) (Table 2). In conclusion, none of the signals related to susceptibility was clearly confirmed to be of significance when tested with univariate correlation tests.

2.2.5. Correlations between Chemical and Morphological Characteristics Related to Thrips Resistance

All of the metabolites related to resistance were strongly correlated with each other. In addition, they were strongly correlated with the density of papillae. Remarkably, none of the compounds that were associated with susceptibility were correlated with the density of papillae. However, the high correlation amongst compounds prevents the use of multiple regression. The highest-explained variance for thrips resistance was explained by the density of papillae, alanine, threonine, and the compound related to signal B (δ 0.90).

3. Discussion

Gladiolus varieties showed a broad range of variation in thrips, resistance as demonstrated by the a more than 130-fold difference in silver damage between the most resistant and the most susceptible variety. Such a large variation is not uncommon for ornamentals. Chrysanthemum varieties also exhibit around 100-fold variation in thrips damage [33,34]. Gaum et al. [35] observed that variation in thrips resistance was six times lower in resistant varieties compared with susceptible ones in a study on 25 rose varieties.

The density of papillae was negatively correlated with thrips damage, while the length of mesophylls and epidermal cells was positively correlated with thrips damage. As a rule, an epidermal cell produces one papilla. Thus, varieties with shorter leaf cells have a higher density of epicuticular papillae. Statistically, it is not possible to distinguish between the effects of cell length and density of papillae on silver damage. Papillae may inhibit the movement of thrips or hinder penetration of the epidermis while feeding. However, Prüm et al. (2013) reported that papillae may slightly enhance adhesion to leaves in the Colorado beetle. In line with our study, Scott Brown and Simmonds [36], who studied the effects of leaf morphology on *Heliothrips haemorrhoidalis*, reported that this thrips has a preference for leaves with smooth surfaces, while trichomes and leaf surface wax structures inhibit thrips. Trichomes were also implicated to be related to thrips resistance in tomato [37] and chili peppers [38].

Besides forming a physical barrier, papillae may store plant secondary compounds. The epidermal papillae of *Pandanus amaryllifolius* Roxb. are the storage site of the basmati rice aroma compound, 2-acetyl-1-pyrroline [11]. Cardinosin A, an aspartic proteinase, suggested to be involved in plant defense against pathogens, is stored in the stigmatic papillae of *Cynara cardunculus* L. [12]. Similarly, Gladiolus varieties with higher densities of papillae may contain higher amounts of defense compounds. The density of papillae explained 39% of the variation in silver damage. The correlation between papillae density and thrips damage shows that the density of papillae sets an upper limit to silver damage. However, other factors may be involved as well, as could be seen from the two varieties with low silver damage but relatively low density of papillae. This leads to false negatives when this morphological marker is the only marker used in breeding programs for thrips resistance in Gladiolus. In addition to papillae, chemical traits are likely candidates to be involved in thrips resistance.

We showed that variation in the plant's metabolome caused variation in thrips mortality in in vitro bioassays. This variation was highly correlated with thrips damage in the whole-plant bioassays. We identified two amino acids and two triterpenoid saponins that were associated with thrips resistance by correlating their relative leaf concentrations with thrips resistance of varieties differing in papillae density. All the compounds that were correlated with resistance were highly correlated amongst each other as well as with papillae density. Remarkably, no compound was clearly related to thrips susceptibility in univariate analyses. One explanation for the combination of these results could be that papillae are involved in resistance to thrips by producing or storing the compounds causing resistance. If this, indeed, is the case, it would also suggest that the physical effect of papillae on thrips resistance is relatively small because, e.g., threonine explains slightly more of the variation in thrips resistance than the density of papillae. Threonine

explained 40% of the variation in silver damage. However, the strong correlation among factors identified as being associated with thrips resistance makes it difficult to separate their effects from each other. Likewise, due the correlations between compounds as well as metabolites and density of papillae, no particular single compound can be pinpointed to be related to thrips resistance. Further study on how papillae deter thrips by producing or storing metabolites related to resistance is necessary. Papillae as storage sites of plant defense secondary compounds have been reported in rice [11] and cardoon [12]. In addition to papillae, it is known that compounds produced in leaf trichomes, such as acylsugars, contribute to thrips resistance in wild tomato [9]. Tomato lines bred with increased amounts of acylsucrose show decreased oviposition by western flower thrips and suppressed inoculation with tomato spotted wilt virus [39]. Although tomato trichomes have been reported to be rich in terpenes, containing up to eight different monoterpenes, none of the trichome exudates are related to thrips resistance [26]. It should be pointed out, however, that from our correlative studies, we cannot exclude an alternative hypothesis. For instance, a low density of papillae could result in higher feeding levels, which, in turn, leads to a rapid induction of defense metabolites. If this hypothesis were true, this could also cause a correlation between defense metabolites and papillae density. A logical follow-up of our study would be to analyze the metabolites in the papillae themselves rather than in the whole leaf. This can be further examined by histochemical studies or by analyzing the expression of genes that encode the committed steps in the synthesis of triterpenoid saponins [40]. This offers a promise for further research on the mechanisms involved in resistance. In addition, it should be noted that the factors we identified only explain 40% of the variation in thrips resistance. Most likely, other factors play an additional role in the defense of Gladiolus against thrips. We used NMR analyses. While this has the advantage of an unbiased approach, the sensitivity is relatively low, which may have resulted in important metabolites with respect to defense not being detected.

Both saponins and the amino acids alanine and threonine have been mentioned in the literature in relation to resistance to insect herbivores. The concentration of alanine was higher in a peach variety resistant to the Mediterranean fruit fly compared with a susceptible variety, while for threonine, such a difference was not detected [41]. In addition, Leiss et al. [27] reported that alanine and threonine occur in higher concentration in the leaves of thrips-resistant carrots, leading to higher thrips mortality. In contrast, Dillon and Kumar [42] reported that the concentration of threonine is significantly higher in *Sorghum bicolor* seedlings resistant to the stem borer *Chilo partellus* than in the seedlings of a susceptible variety, while alanine concentrations do not significantly differ. These results confirm the notion that these amino acids may be involved in thrips resistance. However, the correlation we determined for the two amino acids alanine and threonine does not necessarily mean that these compounds confer resistance to thrips. Biosynthetically, these amino acids share the precursor acetyl CoA with the pathway of triterpenoid saponins CoA [43]. It is likely that that these amino acids are associated with a pool of metabolites that support the synthesis of compounds such as triterpenoid saponins, which may act as resistance metabolites. More detailed metabolomic studies, including fluxomics, can shed more light on this.

Saponins are well known to infer resistance to plant herbivores. Saponins were shown to be important defensive chemical in *Aesculus pavia* against the leafminer *Cameraria ohridella* [44]. This leafminer causes heavy damage to the white-flowering horse chestnut in Europe. Among the Aesculus genus, *A. pavia* L., an HBT genotype, characterized by red flowers, showed an atypical resistance toward this pest. This resistance appeared to be based on exogenous saponins that were translocated from roots/stem to the leaf tissues. Saponins have been reported to mediate the resistance in *Barbarea vulgaris* and counter adaptations in the flea beetle *Phyllotreta nemorum* [45,46]. Higher concentrations of triterpenoid saponins in *B. vulgaris* increased resistance to the diamondback moth *Plutella xylostella* as well as western flower thrips, resulting in significantly

fewer adults and larvae [47]. Saponins from resistant varieties of garden pea inhibited development of the Azuki bean beetle *Callosobruchus chinensis*, whereas saponin extracts from non-resistant legumes did not [48]. The mechanism through which saponins contribute to resistance are largely unknown. Ishaaya [49] suggested that they slow down the passage of food through the gut, whereas Shaney et al. [50] suggested that saponins block the uptake of sterols, an essential compound that insects cannot synthesize but have to take up through feeding. De Geijter et al. [51] reviewed the effects of saponins on insect herbivores and concluded, "These interesting plant compounds offer new strategies to protect crops in modern agriculture and horticulture with integrated pest management (IPM) programs against pest insects, either by spraying or by selecting high-saponin varieties of commercial crops."

Our study indicates that both some chemical compounds and papillae density show a strong negative correlation with feeding damage by thrips; however, correlation does not mean causation. Thus, other associated characteristics may be involved in the mechanism of resistance. Meanwhile, papillae density may provide an easy marker in Gladiolus-breeding programs targeted at increased resistance to thrips.

4. Materials and Methods

4.1. Plant Materials

Fourteen different Gladiolus varieties differing in size were used. Six small varieties (Charming, Charming Beauty, Nymph, Alba, Elvira, and Robinetta) were obtained from Gebr P. & M. Hermans (Lisse,The Netherlands) and eight medium-to-large size varieties (Ben Venuto, Red Balance, V-29, Chinon, Live Oak, Deepest Red, Green Star, and Essential) were obtained from VWS B.V. (Alkmaar, The Netherlands). Each bulb was planted into a 9×9 cm^2 pot filled with a 1:1 mixture of potting soil and dune sand. Six to ten replicates of each variety were randomly placed in a growth room (L:D, 18:6, 20 °C) and grown for 10 weeks. Three to five replicates of each variety were used for a whole-plant thrips bioassay, while the remaining replicates were used for measuring morphological parameters and metabolomic analysis.

4.2. Plant Resistance to Thrips

A non-choice whole-plant bioassay was conducted, as described in Leiss et al. [25]. Plants were placed individually in a thrips-proof cage, consisting of a plastic cylinder (80 cm height, 20 cm diameter), closed with a displaceable ring of thrips-proof gauze. The cages were arranged in a fully randomized design in a climate chamber (L18: D6, 20 °C). Next, 2 male and 18 female adult western flower thrips were added and left for 2 weeks. Thereafter, silver damage, expressed as the leaf area damaged in mm^2, was visually scored for each plant.

4.3. Morphological Measurements

Morphological resistance traits were measured on the longest leaf of each replicate. We measured the length of the leaves, length of epidermal cells and mesophylls as well as the density of the epicuticular papillae, which form a convex outgrowth of the epidermal cells. The density of papillae was measured as the number of papillae per 2100 μm^2. To measure these traits, cross sections of fresh leaves were examined under a confocal laser scanning and a visual light microscope (Zeiss LSM Exciter) with 20× magnification. Measurements were conducted using ImageJ software. To visualize the leaf surface of Gladiolus varieties, we selected Charming Beauty and Robinetta as representatives of a variety with high and low thrips damage, respectively, for scanning electron microscopy (SEM). We used a JSM6400 scanning electron microscope (JEOL; Tokyo, Japan). Leaf discs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7), followed by dehydration with a graded series of acetone solutions (70%, 80%, 90%, 96%, and 100% acetone) for 10 min each. Before imaging, specimens were oriented, mounted on metal

stubs, and sputter-coated with gold (Polaron 5000 sputtering system). In addition, the plant dry mass was measured after drying plants for 3 days in an oven at 50 °C.

4.4. In Vitro Thrips Bioassay

We first conducted an in vitro thrips bioassay using leaf extracts to investigate the potential effects of plant defense compounds. For this bioassay, we used 6 varieties that differed in susceptibility in the whole-plant bioassay described: Charming, Charming Beauty, Nymph, Alba, Elvira, and Robinetta. Briefly, 50 mg of dried leaf material of five replicates per variety was weighed and pooled for chemical extraction. The samples were extracted with 50% methanol in an ultrasonic bath for 20 min. After filtration, the residue was extracted again. The extraction was repeated three times, and the final filtrate was dried in a rotary evaporator. Next, 9 mg/mL of these extracts was re-dissolved in a 5% methanol–water solution, and the pH was adjusted to 7 [27].

For the in vitro thrips bioassay (Figure S2), 96-well plates were filled with 150 of µL 2% agarose and 50 µL of the extracts to be tested. Methanol—water (5%) and the insecticide abamectin (50 µg/mL) were used as a negative and a positive control, respectively. Each bioassay consisted of 32 replicates with 1 column of 8 wells on each of 4 plates. A single first instar thrips larva was placed into each cap of an 8-cap flat-cap strip. Each cap was sealed with parafilm through which the thrips could feed. The strips were then placed on top of the 96-well plates. All the wells in one column of the 96-well plates received the same treatment. One variety, therefore, consisted of 8 replicates. Each variety was added to columns of 4 different 96-well plates, thus yielding 32 wells for each variety. An adhesive sealing film was placed onto the plates to prevent evaporation and to protect the samples during the assay. All plates were placed up-side down for 48 h to ensure that the thrips got into contact with the extracts. The plates were randomly placed in a growth chamber with standard thrips-rearing conditions (L18: D6, 23°C, 65% RH). After 48 h, the mortality of the thrips was recorded. Differences in thrips mortality among varieties were statistically analyzed with a chi-square test. The correlation between thrips mortality in the in vitro bioassay and thrips silver damage in the whole-plant non- choice bioassay was analyzed using Pearson correlation tests.

4.5. Metabolic Profiling

4.5.1. Extraction of Plant Materials for NMR Metabolomics

Three replicates of leaves of each of the fourteen varieties were used for NMR metabolomics. The standard protocol of sample preparation and ^{1}H NMR profiling described by Kim et al. [52] was applied. Samples of 30 mg of freeze-dried plant material were weighed into a 2 mL microtube and extracted with 1.5 mL of a mixture of phosphate buffer (pH 6.0) in deuterium oxide containing 0.05% trimethylsilylproprionic acid sodium salt-d_4 (TMSP) and methanol-d_4 (1:1). Samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13,000 rpm for 10 min. An aliquot of 0.8 mL of the supernatant was transferred to 5 mm NMR tubes for ^{1}H NMR measurement.

4.5.2. NMR Analysis

^{1}H NMR spectra were recorded with a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Deuterated methanol was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans, requiring 10 min and 26 s acquisition time, with the following parameters: 0.16 Hz/point, pulse width (PW) of 30° (11.3 µs), and relaxation delay (RD) of 1.5 s. A pre-saturation sequence was used to suppress the residual water signal with low-power selective irradiation at the water frequency during the recycle delay. Free induction decay (FID) was Fourier-transformed with a line broadening (LB) of 0.3 Hz. The resulting spectra were manually phased and baseline-corrected to the internal standard TMSP at 0.00 ppm using TOPSPIN version 3.5 (Bruker). Two-dimensional J-resolved NMR spectra were acquired using 8 scans per 128 increments for F_1 (chemical shift axis) and 8 k for F_2

(spin–spin coupling constant axis) using spectral widths of 66 and 5000 Hz, respectively. Both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double-complex Fourier transformation. J-resolved spectra were tilted by 45°, symmetrized about F_1, and then calibrated to TMSP using XWIN NMR version 3.5 (Bruker). ^{1}H–^{1}H-correlated COSY spectra were acquired with a 1.0 s relaxation delay and a 6361 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 0).

4.5.3. Data Processing

For the NMR spectra, intensities were scaled to total intensity and reduced to integrated equal widths (0.04 ppm) corresponding to the region of δ 0.32–10.0 (Table S1). The regions of δ 4.7–5.0 and δ 3.30–3.34 were excluded from analysis due to the presence of the residual signals of water and methanol. ^{1}H NMR spectra were automatically binned by AMIX software version 3.7 (Biospin, Bruker). Data were further analyzed with principal component analysis (PCA) and orthogonal partial least squares–discriminant analysis (OPLS-DA) using SIMCA-P software version 12.0 (Umetrics, Umea, Sweden). One of unsupervised multivariate methods is principal component analysis (PCA). It is used to reduce the dimensionality of a multivariate dataset. However, there is a limitation to see minor separation, because PCA could extract grouping information only from the maximum separation on the signals in the spectra representing the metabolomes. To solve the limitation of PCA, a supervised multivariate data analysis, orthogonal partial least squares (OPLS)—discriminant analysis (DA), in which another dataset of resistance was correlated with the chemical dataset, was performed. Pareto scaling was used for PCA and unit variance scaling for OPLS-DA.

4.6. Statistical Analysis

Differences between varieties in morphological traits and plant dry mass were analyzed with one-way ANOVA and subsequent post hoc analysis with Bonferroni correction. Silver damage did not fit a normal distribution and was, therefore, ln-transformed. Correlations between thrips silver damage and morphological traits were analyzed using Pearson correlation.

Differences between Gladiolus varieties in the relative concentrations of metabolites related to thrips resistance were analyzed by one-way ANOVA. Pearson correlations were calculated for the relationships between metabolite concentrations, thrips silver damage, and density of epicuticular papillae. For epicatechin, epigallocatechin, and gallic acid, Kruskal–Wallis and Spearman rank correlations were used because data were not normally distributed.

5. Conclusions

Our study suggests that chemical compounds produced or stored in the epidermal papillae may confer thrips resistance to Gladiolus species. This offers an existing promise for further research on the mechanisms involved. Meanwhile, the density of papillae may provide an easy marker in Gladiolus-breeding programs targeted at increased resistance to thrips.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10071384/s1, Figure S1: PCA score plot for 14 varieties based on thrips resistance of the varieties. (▲) susceptible varieties, (●) medium varieties and (■) resistant; Figure S2: The in-vitro thrips bioassay; 96-well plates at top view (A) and at side view (B); Table S1. Binning table of 14 Gladiolus varieties from ^{1}H NMR spectra.

Author Contributions: P.G.L.K. and K.A.L. designed the study. D.S.C.W. implemented the bioassay experiments and sampling and carried out data analysis and interpretation. D.S.C.W. and Y.H.C. performed the NMR analysis. D.S.C.W. prepared the manuscript, which was commented on by P.G.L.K., K.A.L. and Y.H.C. All authors have read and agreed to the published version of the manuscript.

Funding: The first author holds a grant of the Directorate General of Higher Education (DHGE) of the Republic of Indonesia to do the study.

Data Availability Statement: ^{1}H NMR data of the samples are deposited in the data storage of Natural Products Laboratory (Institute of Biology, Leiden University, Leiden, The Netherlands). The data can be provided upon request.

Acknowledgments: We thank the Dutch Gladiolus breeders Gebr. Hermans and VWS B.V. (Alkmaar, the Netherlands) for providing the different Gladiolus varieties. Gerda Lamers is thanked for her technical assistance in microscopy. Suzanne Kos, Rita Rakhmawati, and Mariá José Rodriguez-Lopez from Plant Sciences and Natural Products, Institute of Biology (IBL), Leiden University, are thanked for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Sown Wildflowers Enhance Habitats of Pollinators and Beneficial Arthropods in a Tomato Field Margin

Vaya Kati [1,*], Filitsa Karamaouna [1,*], Leonidas Economou [1], Photini V. Mylona [2], Maria Samara [1], Mircea-Dan Mitroiu [3], Myrto Barda [1], Mike Edwards [4] and Sofia Liberopoulou [1]

1 Scientific Directorate of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, 8 Stefanou Delta Str., 14561 Kifissia, Greece; l.economou@bpi.gr (L.E.); m.samara@bpi.gr (M.S.); m.barda@bpi.gr (M.B.); albilyb@hotmail.com (S.L.)
2 HAO-DEMETER, Institute of Plant Breeding & Genetic Resources, 570 01 Thessaloniki, Greece; phmylona@nagref.gr
3 Faculty of Biology, Alexandru Ioan Cuza University, Bd. Carol I 20A, 700505 Iași, Romania; mircea.mitroiu@uaic.ro
4 Mike Edwards Ecological and Data Services Ltd., Midhurst GU29 9NQ, UK; ammophila@macace.net
* Correspondence: v.kati@bpi.gr (V.K.); f.karamaouna@bpi.gr (F.K.); Tel.: +30-210-8180-246 (V.K.); +30-210-8180-332 (F.K.)

Abstract: We evaluated the capacity of selected plants, sown along a processing tomato field margin in central Greece and natural vegetation, to attract beneficial and Hymenoptera pollinating insects and questioned whether they can distract pollinators from crop flowers. Measurements of flower cover and attracted pollinators and beneficial arthropods were recorded from early-May to mid-July, during the cultivation period of the crop. Flower cover was higher in the sown mixtures compared to natural vegetation and was positively correlated with the number of attracted pollinators. The sown *Glebionis coronaria*, *Coriandrum sativum*, *Anethum graveolens*, and *Fagopyrum esculentum* attracted mainly wild bees, which were the most abundant pollinating insects. In the natural vegetation, *Rapistrum rugosum* attracted mainly honeybees, while Asteraceae, Convolvulaceae, and Apiaceae species attracted wild bees. Beneficial arthropod abundance and diversity were higher in the sown mixture. Tomato flowers were visited by a small number of wild bees. Their number was not affected by the distance from the field margin, indicating no distraction effect from the sown or natural vegetation flowering plants. Our results suggest that selected flowering plants can improve the field margin habitats for pollinating insects and beneficial arthropods, but more work is needed to elucidate the effect on crop pollination.

Keywords: field margin; flowering plants; Hymenoptera pollinators; beneficial arthropods; crop pollination; processing tomato

1. Introduction

The increasing global demand for food has pushed modern farming systems into setting higher crop productivity goals, leading to intensive cultivation practices [1] and the simplification of the agricultural landscape, with vast monocultures and an over-reliance on agrochemical inputs. However, these practices have been associated with several negative consequences on the functioning of agroecosystems, especially in arable farmland [2]. Intensive cultivation practices in arable fields have led to the deterioration of flora and fauna biodiversity [3], threatening the sustainability of crop production and ultimately undermine the very same goal of food security they were employed to achieve. Among the main agroecosystem services affected is crop pollination, essential for the productivity of 75% of the main food crops [4] with an estimated worldwide value of 153 € billion [5] and biological pest control, with an estimated global value of around 40 € to 74 € billion [6]. Thus, understanding the negative impacts of agricultural

intensification on crop productivity and developing sustainable management strategies to offset them, has increasingly been in the spotlight of research [7–9]. Several studies have addressed the loss of habitats for pollinating insects and beneficial arthropods in intensive farming systems and the associated decline in pollination [10,11] and biological control services [12,13], while others outlined the complex, mutually beneficial interaction between weeds and insect pollinators in the agricultural landscape [14,15].

Many European countries, especially central and north, have widely adopted the practice of sowing selected plant species to hinder the deterioration of habitats for pollinators and beneficial arthropods and support the interrelated multitrophic systems [16]. Purpose-designed schemes, providing economic incentives for farmers to implement agro-environmental measures, such as appropriate management of field margins, have contributed to the wide implementation of these practices [2,17,18]. Plant mixtures, comprising annual and perennial species, have been studied for their role in supporting pollinators, beneficial arthropods, and other invertebrates in various crops and landscape scenarios [19–21]. However, the practice of field margin management is not commonplace in southern European countries, like Greece, where the small field size, measuring on average 6.6 ha [22] means that, to maximise economic returns, the available land is cultivated from end to end. Consequently, the information on flora and fauna biodiversity and their ecosystem services is limited and is available only for the perennial olive orchards [23–25].

Field grown tomato (*Solanum lycopersicum*) is self-pollinated or partially dependent on flower-visiting insects for effective pollination and fruit production [4]. Knowledge on the contribution of insects, especially wild bees, for the pollination of processing tomato remains limited [26]. Greenleaf and Kremen [26] demonstrated that, although the field-grown tomato is regarded as mainly self-pollinated, its production was substantially increased under pollination by wild bees such as *Bombus* and *Anthophora* species, and suggested that cross-pollination may enhance tomato fruit-set over self-pollination, especially in tomatoes grown under high temperatures which can cause pollen sterility. The exent of this phenomenon is variable and depends on the position of the flowers on the plant, thus cross-pollination can increase the chances of fertile pollen reaching more flowers [26]. According to Teppner [27], pollinators on open cultivated tomato in Central Europe, which benefit the pollination of tomato due to their 'buzzing' vibration effect on the flowers, included the *Bombus* species *B. pascuorum* and *B. terrestris*, *Megachile willughbiela*, *Hylaeus gibbus* and buzzing *Lasioglossum* species. Therefore, bees associated with the pollination of field grown tomato are important for crop production and their communities should be maintained with the support of suitable habitats that provide food and shelter [26]. Moreover, the presence of non-crop flowering plant habitats could also contribute to the conservation of parasitoids and generalist natural enemies that ultimately could suppress tomato pests and reduce crop damage [19,28–30]. None the less, field margin plants and arthropod communities' interaction is complex and can vary greatly depending on plant species composition [31]. For example, some plant species could provide resources not only to natural enemies but also to pests, shifting the balance towards herbivorous populations and eventually leading to crop damage [32]. In addition, plant species which are attractive to natural enemies or pollinating insects could act either as a reservoir that would benefit the crop [33] or as a distraction for these insects from the crop plants.

Here, we questioned whether selected flowering species sown in the field margin of a processing tomato located in one of the main areas for this crop in Greece, can establish successfully and create a habitat that would increase the abundance and diversity of pollinating insects and beneficial arthropods in the field margin compared to natural vegetation of arable weeds and examined whether this practice could have a distraction effect for the pollinators visiting crop plants. The current study was conducted in the frame of the biodiversity project Operation Pollinator, implemented in various crops in Greece since 2010 [23]. The aims of the project follow the principles of the EU policy for sustainable agriculture, dictated through the new common agriculture policy (CAP)

and the Directive (2009/128/EC) for the sustainable use of plant protection products, as it supports the protection and enhancement of flora and fauna biodiversity in the agro-ecosystems.

2. Results

2.1. Sown Plants Establishment and Flowering

Flowering of the winter mixture (WM) started around mid-April and lasted until mid-July (Figure 1 and Supplementary Figure S1). Species reached flowering in the successive order of *Vicia villosa* = *Lathyrus sativus* > *Glebionis coronaria* > *Coriandrum sativum* > *Anethum graveolens*. The emergence of the remaining sown species, *Tagetes patula* and *Matricaria chamomilla*, was in small numbers (<1 plant/m^2) and their flowering had negligible contribution. The Fabaceae species were the earliest to reach flowering which lasted until early May, while *G. coronaria*, which started flowering around early-May, had the longest lasting flowering period (more than eight weeks with a peak towards the end of May) compared to all the other species. Both sown Apiaceae species started flowering around mid-May. Arable weeds that emerged together with the sown ones in WM included the species *Sonchus oleraceous*, *Picris echioides*, *Lactuca serriola*, *Cichorium intybus*, *Veronica hederifolia*, *Sinapis arvensis*, *Papaver rhoeas*, *Daucus carota*, and *Convolvulus arvensis*. A two-way ANOVA was carried out on the % flower cover by type of winter treatment (sown WM and natural vegetation sites NV1 and NV2) and time (five sampling dates, from May to July) (Supplementary Table S1). The main effects of treatment type and time, and their interaction were significant. Overall, the sown WM had significantly higher flower cover than either the NV1 or NV2 site. The difference was significant from late-May to mid-July, while in early-May the flower cover between the WM and the NV2 site was similar.

In the summer mixture (SM), *Fagopyrum esculentum* established successfully and reached flowering from mid-June to mid-July, while *Phacelia tanacetifolia* emerged in small numbers and had limited contribution to the percentage of flower cover (Figure 1). *Petroselinum crispum* did not emerge. The SM plots had also a low density of arable weeds (dicotyledons *Convolvulus arvensis*, *Picris echioides*, *Solanum nigrum*, *Abutilon theophrasti*, *Amaranthus* sp., and monocotyledons *Sorghum halepense*, *Echinochloa crus-galli*, *Digitaria* sp., and *Cyperus* sp.). A two-way ANOVA was carried out on the % flower cover by type of field margin treatment (including both sown mixtures SM and WM and the natural vegetation sites NV1 and NV2) and time (two sampling dates in mid-June and mid-July, when SM and WM had synchronous flowering). The main effect of treatment was significant, while the effect of time and their interaction was not significant (Supplementary Table S1). The SM had significantly higher flower cover compared to either of the NV sites and the WM.

The flowering in the two natural vegetation sites NV1 and NV2 was provided by different species (Supplementary Figure S2). These mainly were *Picris echioides*, *Daucus carota*, *Anthemis altissima*, and *Convolvulus arvensis* in the NV1 site, and *Rapistrum rugosum*, *Scabiosa atropurpurea*, *Convolvulus arvensis*, and *Cichorium intybus* in the NV2 site (Figure 1). Other flowering species emerging outside the designated NV plots, such as *Matricaria chamomilla* (Asteraceae) early in the season and *Galega officinalis* (Fabaceae) at the banks of a neighboring draining channel in July, were recorded, though not included in the measurements.

The tomato crop was in flower from June to July and was harvested at the end of July. The economic crop flowering, i.e., the flowers that produced the fruits harvested, were those pollinated before mid-July, while any fruits produced after that period were not of economic value for the farmer (they were not harvested). Therefore, the economic crop flowering coincided with the end of flowering of the WM and the peak of flowering in the SM.

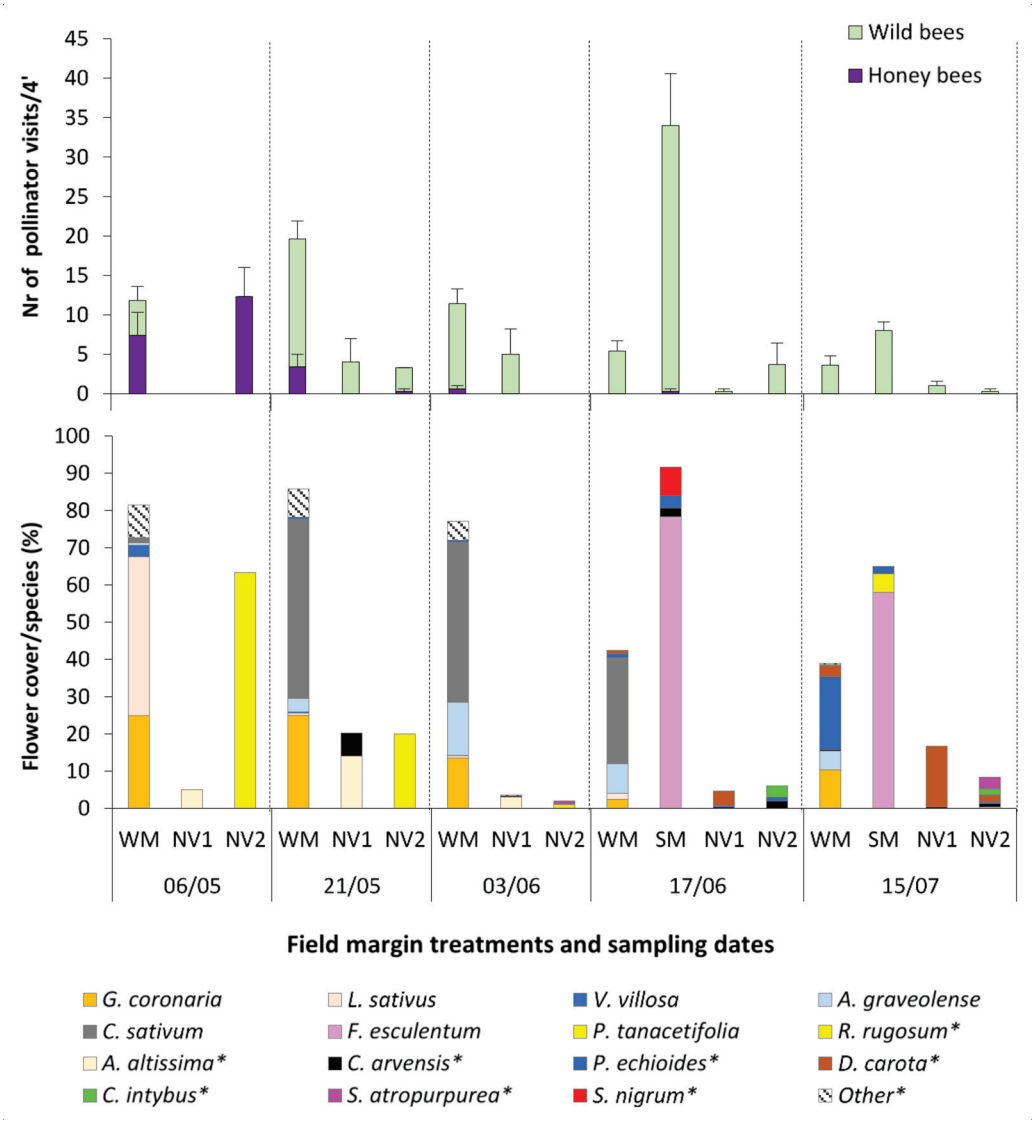

Figure 1. Mean flower cover percentage per species in the winter mixture (WM), spring mixture (SM) and two natural vegetation sites (NV1 and NV2), and corresponding mean numbers of Hymenoptera pollinator visits (wild bees and honey bees) recorded for 4′/14 m^2 plot, at five sampling dates. Flowering species in the natural vegetation and flowering weeds that emerged in the plots with the sown mixtures are noted with an asterisk (*). Other species: *Sonchus* sp., *S. arvensis*, *P. rhoeas*, *L. serriola*, *V. hederifolia*, *Lathyrus* sp. Vertical bars represent standard error of means.

2.2. Effect of Wildflower Margins on Pollinator Abundance and Diversity

The Hymenoptera pollinators were honeybees early in the season and predominantly wild bees thereafter (Figure 1). Bumble bees or megachilids were not present at any sampling date. The total number of Hymenoptera pollinators visiting the flowers of the sown or the natural vegetation plants in the margins and the corresponding percentage of flower cover in each site had a positive correlation (R^2 = 0.58) (Figure 2).

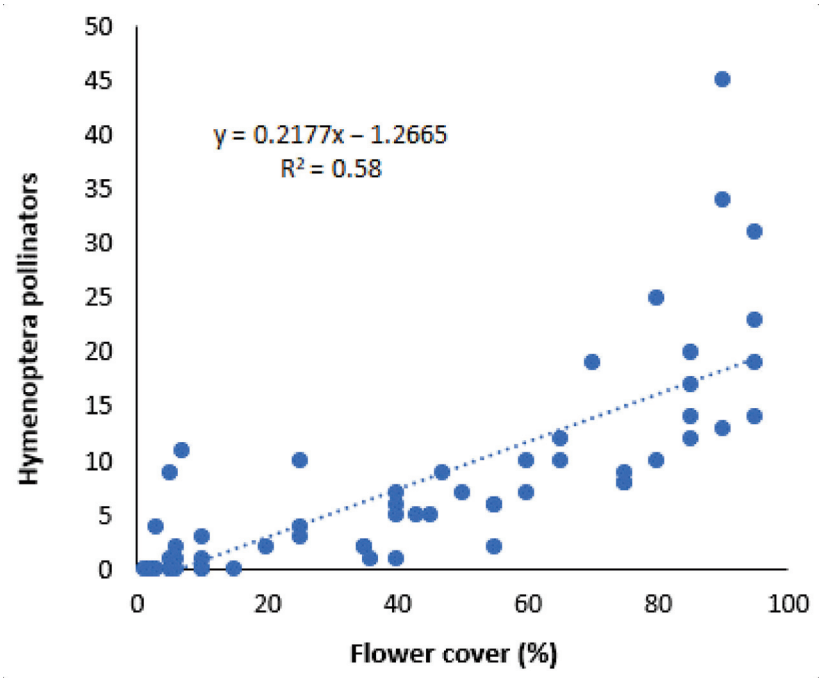

Figure 2. Correlation of flowering cover (%) in the field margins with sown or natural vegetation species and mean number of Hymenoptera pollinators (wild bees and honey bees) recorded at five sampling dates during May–July.

The type of treatment (WM or SM vs. natural vegetation margin) and the sampling time had a significant effect on the number of wild bees and pollinators in total (2-way ANOVA, $p = 0.05$; Supplementary Table S2) while their interaction was not significant. The WM field margin attracted significantly higher number of total pollinators (10 visits/plot/4′) compared to the NV1 and NV2 plots (two and four visits/plot/4′, respectively) while higher number of pollinators was recorded in late-May to early-June, compared to that in mid-June to mid-July, which is in line with the decline in flowering recorded after mid-June in the winter sown species (Figure 1 and Supplementary Table S2). The SM field margin also had significantly higher number of total pollinators (21 visits/plot/4′), compared to either the NV1 or NV2 site (0.3 and two visits/plot/4′, respectively), with significantly more visits recorded in mid-June compared to mid-July (Figure 1 and Supplementary Table S2). The number of wild bee visits recorded at the SM margin in mid-June was the largest recorded across all measurements in any margin type and sampling date (33.7 ± 6.6 visits/plot/4′). Wild bees were highly attracted by *F. esculentum*, which had a flowering peak at that time (Figure 1).

The wild bees associated with the WM plots (*Andrena* spp., *Colletes* sp., *Hylaeus* spp., *Halictus* sp., *Lasioglossum* spp., *Pseudapis* sp., *Sphecodes* sp., and a few *Eucera* sp.) were observed to forage on *G. coronaria*, *C. sativum* and *A. graveolens*, throughout the flowering period of these species (Supplementary Table S3). *Fagopyrum esculentum* in the SM plots attracted mainly *Andrena* sp. and *Halictus* sp. (Supplementary Table S3). On the other hand, honeybees (*Apis mellifera*) were mainly recorded on the flowers of *L. sativus* in early-May and in smaller numbers on *C. sativum* later that month (Figure 1). The flowering plants in the NV plots attracted wild bees throughout all measurements, with the exception of *R. rugosum* in the NV2 plots, which was mainly foraged by honeybees when it was in full flower (early-May) (Figure 1). In late-May, towards the end of *R. rugosum* flowering, honeybee numbers dropped significantly, while the main pollinators recorded were small numbers of wild bees. Other flowering species of the NV plots that attracted wild bees were *Anthemis altissima* (Asteraceae), *Picris echioides* (Asteraceae), *Daucus carota* (Apiaceae) and *Scabiosa atropurpurea* (Dipsacaceae). The wild bee specimens associated with the NV plots included mainly *Andrena* spp.

The field margins attracted also non-Hymenoptera insects that could contribute generally to pollination. In the WM, these were Diptera (Syrphidae, Stratiomyidae (*Odontomyia* sp.)) and Lepidoptera on *G. coronaria*, and Diptera (Syrphidae) on *L. sativus*, and in the SM Diptera (mostly Syrphidae, other flies) and Lepidoptera on the flowers of *F. esculentum.* Finally, although not included in the measurements, it is noteworthy that *Galega officinalis* recorded in the natural vegetation outside the designated plots in mid-July, was the only flowering species that attracted *Bombus* sp.

The pollinators observed on the crop flowers during the two sampling days (17 and 23 June) were only wild bees, while no honeybees or other Apidae were present. The number of wild bees was not affected by the distance between the tomato crop row and the sown margin and remained very low (1–2 individuals in total of three replications, in each sampling site).

2.3. Effect of Wildflower Margins on Arthropod Abundance and Diversity

Suction sampling from the sown mixtures and the natural vegetation sites resulted in a total of 2256 individuals, belonging to over 50 families of Insecta and Arachnida taxa (Supplementary Table S4). Beneficial arthropods included mostly Hymenoptera parasitoids, generalist or specialist insect predators and spiders. The field margin with winter treatment (WM and NV) and sampling time had a significant effect on the Shannon diversity index (*H*) of total arthropods and Hymenoptera parasitoids (families) recorded in the suction samples (two-way ANOVA, $p = 0.05$; Table 1). The *H* index of total arthropods in the WM (2.26) was significantly higher compared to the native vegetation plots NV1 (1.90) and NV2 (2.04) (Table 1). On the other hand, the *H* index of the Hymenoptera parasitoids was significantly higher in the WM (1.34) compared to NV1 (0.93) but similar to NV2 (1.05), while it was significantly lower at the early-June measurement. Interestingly, despite the progressively lower flower cover of the WM with time, the *H* index of total arthropods remained significantly higher for this mixture (2.58) than the SM (2.19), with the latter being similar to the NV1 (2.21) and NV2 (2.19) sites (Table 1).

The main effect of field margin treatment (either for the winter treatments WM and NV1, NV2 or for both the winter and summer treatments WM, SM, NV1, NV2) and of time was significant on the abundance of Hymenoptera parasitoids and predators. The interaction between field margin type and time was not significant. The low numbers of parasitoids recorded in the WM plots at the early-June measurement compared to previous and later samplings, could be due to a possible temporary negative effect from pesticide application to the crop the day before the sampling.

The WM samples contained significantly more parasitoids and predators compared to any other field margin treatment (including SM) across all sampling times (Figure 3 and Supplementary Table S5). The overall mean (±s.e.m.) of natural enemies across the winter treatments was 35.1 (±5.2) parasitoids and 40.6 (±4.9) predators in the WM, while the corresponding means in the NV1 samples were 11.7 (±2.9) and 13.6 (±3.2) and in the NV2 were 11.6 (±2) and 14.7 (±2.2) for parasitoids and predators, respectively. The highest number of natural enemies was recorded in the WM at the mid-July measurement, with 56.4 (±6.8) parasitoids and 59.6 (±5.8) predators, respectively. At that time, despite coinciding with the peak of *F. esculentum* flowering, the samples from the SM plots contained only 4.7 (±2.2) parasitoids and 12 (±2.1) predators (Supplementary Table S5). Correlation analysis between the beneficial arthropod abundance and the percentage of flower cover revealed no association ($R^2 = 0.02$). The relationship between the beneficial arthropods and the flowering species diversity (*H*) was more evident, although still weak ($R^2 = 0.18$).

Table 1. Shannon diversity Index (*H*) of total arthropods and Hymenoptera parasitoids (mean ± s.e.m.) in 1′ suction samples/plot with sown selected flower plants or with natural vegetation at the margins of a processing tomato crop, from late-May to mid-July.

Field Margin	Shannon's Diversity Index (*H*)											
	Total Arthropods						Hymenoptera Parasitoids					
	WT				Mean 21/5–15/7	Mean 17/6 &15/7	WT				Mean WT/Margin (21/5–15/7)	Mean WST/Margin (17/6 & 15/7)
	WST						WST					
	21/5	3/6	23/6	15/7			21/5	3/6	23/6	15/7		
WM	1.96 ± 0.06	1.91 ± 0.04	2.57 ± 0.08	2.60 ± 0.07	2.26 ± 0.08 A	2.58 ±0.05 A	1.77 ± 0.18	0.59 ± 0.34	1.35 ± 0.21	1.67 ± 0.09	1.34 ± 0.15 A	1.51 ± 0.12
NV1	1.51 ± 0.25	1.66 ± 0.29	2.46 ± 0.03	1.96 ± 0.16	1.90 ± 0.14 B	2.21 ±0.13 B	0.94 ± 0.23	0.23 ± 0.23	1.70 ± 0.22	0.84 ±0.26	0.93 ± 0.19 B	1.28 ± 0.24
NV2	1.80 ± 0.03	1.97 ± 0.18	2.40 ± 0.11	1.98 ± 0.11	2.04 ± 0.08 B	2.19 ±0.12 B	1.04 ± 0.04	0.66 ± 0.38	1.62 ± 0.14	0.89 ± 0.21	1.05 ± 0.15 AB	1.26 ± 0.20
SM			2.21 ± 0.26	2.16 ± 0.20		2.19 ±0.15 B			16.7 ± 3.4	12.0 ± 2.1		0.89 ± 0.30
Mean WT/date	1.79 ± 0.08 c	1.86 ± 0.09 c	2.49 ± 0.05 a	2.25 ± 0.11 b			1.34 ± 0.15 a	0.51 ±0.19 b	1.52 ± 0.12 a	1.23 ± 0.15 a		
Mean WST/date			2.43 ±0.07 b	2.23 ±0.09 a					0.99 ± 0.53	0.80 ± 0.41		
WT 2-way ANOVA	Mixture: $F_{2,32} = 9.94$, $p = 0.0004$; Date: $F_{3,32} = 21.33$, $p < 0.0001$; Mixture × Date: $F_{6,32} = 1.99$, $p = 0.0966$						Mixture: $F_{2,32} = 3.53$, $p = 0.0413$; Date: $F_{3,32} = 10.16$, $p < 0.0001$; Mixture × Date: $F_{6,32} = 2.12$, $p = 0.0788$					
WST 2-way ANOVA	Mixture: $F_{3,20} = 5.57$, $p = 0.0060$; Date: $F_{1,20} = 6.55$, $p = 0.0187$; Mixture × Date: $F_{6,20} = 2.13$, $p = 0.1288$						Mixture: $F_{3,20} = 1.99$, $p = 0.1475$; Date: $F_{1,20} = 3.71$, $p = 0.0686$; Mixture × Date: $F_{6,20} = 2.46$, $p = 0.0928$					

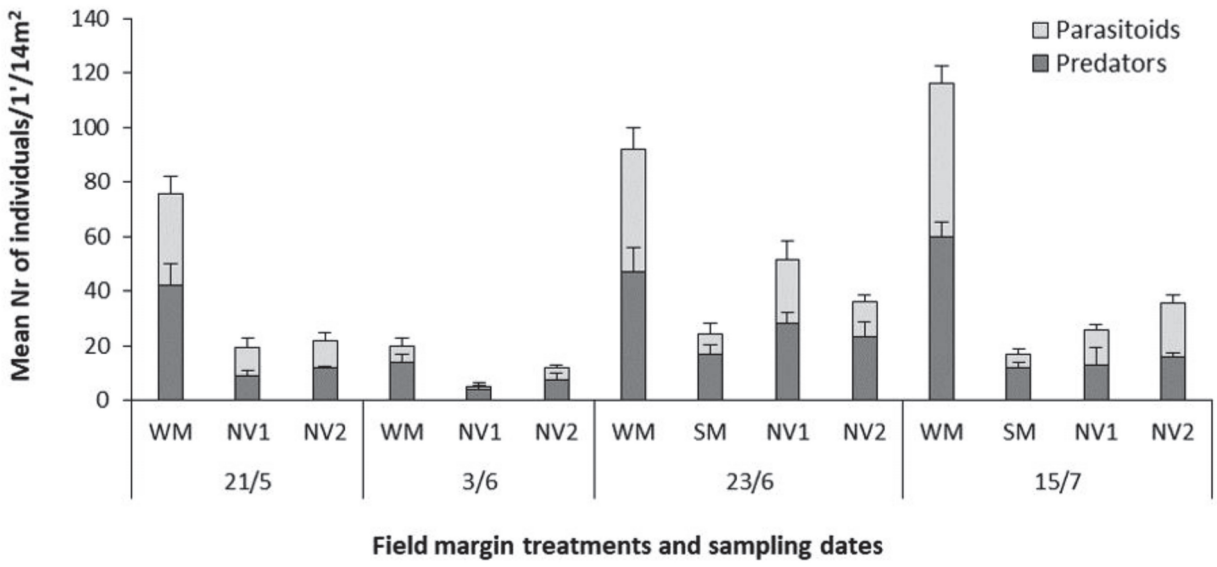

Figure 3. Mean number of parasitoids and predators recorded in suction samples (1′/14 m^2 plot) from the sown (winter mixture WM, summer mixture SM) or natural vegetation (NV) field margins of a processing tomato crop. Vertical bars represent standard error of means.

Parasitic wasps in the samples belonged to six different superfamilies (Ceraphronoidea, Chalcidoidea, Cynipoidea, Diaprioidea, Ichneumonoidea, Platygastroidea) and 18 different families (Figure 4 and Supplementary Table S6). The samples from the WM belonged to 17 families, while the ones from the SM mixture belonged to nine families and from the NV1 and NV2 sites to 13 and 15 families, respectively. Eulophidae and Scelionidae added to more than half of the samples in the WM (approx. 55.5%) while other abundant families were Eurytomidae and Encyrtidae. In the SM, Scelionidae held almost one third of the samples, followed by Eulophidae (17.6%), Eurytomidae (11.8%) and Mymaridae (11.8%). In the natural vegetation plots of both sites, Scelionidae was also the predominant family holding almost 50% of the samples and Eulophidae held another 22–23%. Aphelinidae, Braconidae, and Ichneumonidae, contributing lower percentages in the mixture or the natural vegetation samples, should not be overlooked as they are known families of aphid parasitoids, while other braconids and ichneumonids parasitize larvae of lepidopteran pests. Trichogrammatidae, known to include egg parasitoid species of Lepidoptera larvae, was recorded only on the NV2 plots in the late-June measurement. The sampled insect predators belong to the families Anthocoridae, Chrysopidae, Coccinellidae, Miridae, Syrphidae, and Reduviidae, with the latter found only at natural vegetation NV1 plots (Supplementary Table S4).

Regarding the presence of insect pests on the tomato crop, infestation by the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae) was recorded after visual scouting during the crop season. Its occurrence was common in our experimentation field and within the usual incidence of the pest in the area.

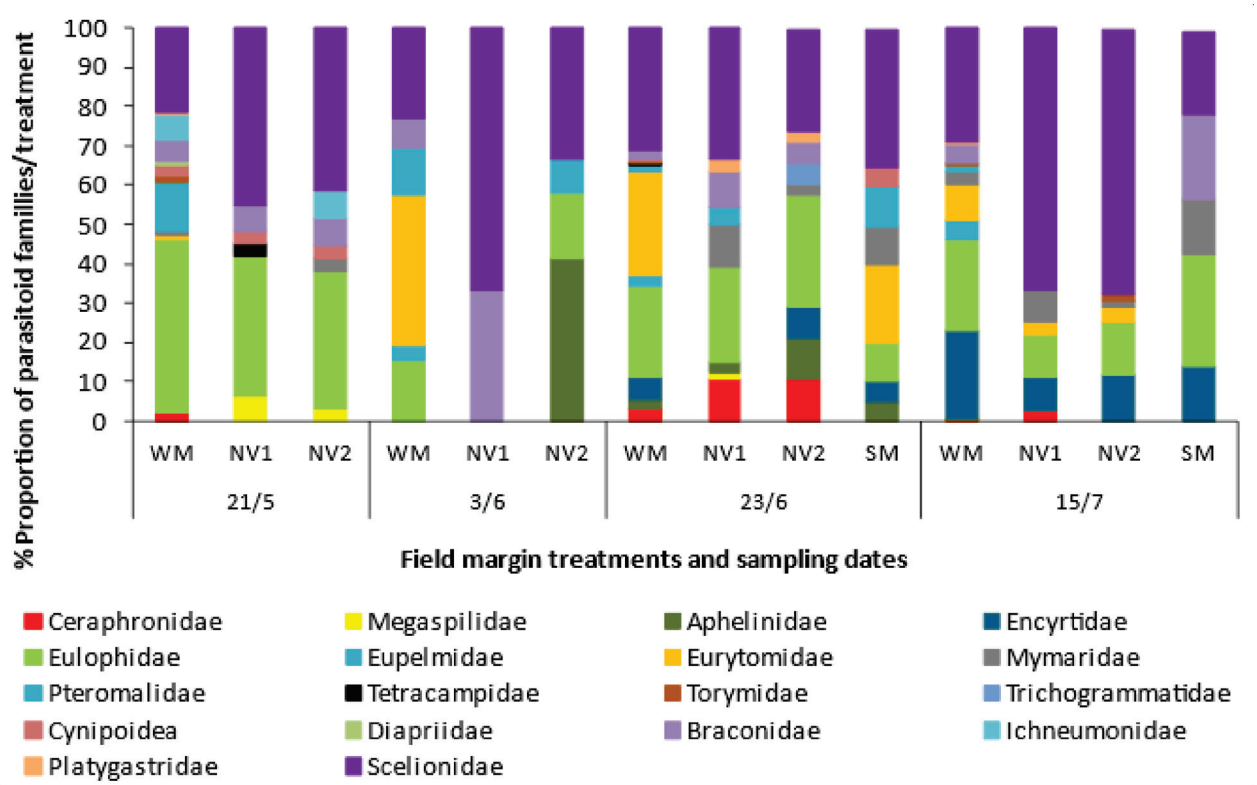

Figure 4. Percentage proportion of parasitoid families in samples of the winter mix (WM), summer mix (SM) and the two sites with natural vegetation (NV1 and NV2), at four sampling dates.

3. Discussion

This work sheds light on the association of plant, pollinator, and beneficial arthropod communities in the field margin of processing tomato, a crop where similar studies are limited. Understanding these associations is the basis of manipulating habitats to augment the ecosystem functions of pollination and biological control. Several biodiversity-ecosystem function studies focus on the manipulation of species richness to measure the effect on an associated ecosystem function [34,35] while others argue that it is probably more important to understand the linkages between key species or functional groups and ecosystem functions [28,36]. The second approach has revealed, in many cases, some level of functional redundancy among species in the provision of certain ecosystem functions [28,37,38]. The selection of different flowering plants and their composition in mixtures to optimize the conservation of arthropod functional groups depends on the suitability of nectar and pollen resources they provide to these groups, on specific plant traits such as floral morphology associated with these groups, and on food preferences and foraging risks for flower visitors [39,40]. Flowering plant mixtures belonging to Apiaceae and other species with exposed floral nectaries are frequently used in biocontrol studies, while pollinator-friendly plant mixtures are often dominated by Fabaceae species [41].

Several studies have suggested that wildflower strips could benefit pollinator diversity [42] by comparing the effect with bare soil. In our study, a more challenging situation was tested as a control, i.e., natural vegetation in the field margins of a processing tomato crop. Although the sown mixtures had comparable or lower species diversity than the natural vegetation at the margins of the crop, they provided higher flower cover throughout the crop season and attracted more Hymenoptera pollinators. The tested WM provided a rich flowering canvas of *L. sativus*, *G. coronaria*, *C. sativum*, and *A. graveolens*, which changed in composition over time, lasting from early-May to mid-July and overlapped with the dense flowering provided mainly by *F. esculentum* in the SM. In terms of conservation of functional pollinator species, the abundance and relative ratio of wild bees and honeybees were associated with the flower species synthesis of the mixture over time, and

outcompeted the natural vegetation. Among the attracted wild bee species, *Hylaeus* spp. and *Lasioglossum* spp. are known to pollinate tomato [27]. Several other wild bees were attracted, which, although not directly involved in the pollination of tomato, included species that are important for their pollination services to other plants of economic or biodiversity value. These included mainly *Andrena* spp., *Colletes* sp., *Halictus* sp., *Pseudapis* sp., and *Sphecodes* sp.

Bumble bees, which forage on *Lathyrus japonicus* [43] and *F. esculentum* [44], and megachilids that also forage on Apiaceae [45], are among the efficient pollinators for tomato. However, in our study neither the WM, which contained *L. sativus* and the Apiaceae *C. sativum* and *A. graveolense*, nor the SM which was dominated by *F. esculentum*, attracted any of these pollinators. The only presence of bumble bees was observed on *G. officinalis* that emerged late in the cropping season in the natural vegetation outside the designated plots. The capacity of *G. officinalis* to attract bumble bees, including *B. terrestris*, has also been reported by Montalva et al. [46].

The main pollinating insects attracted by the flowers of *L. sativus* in the WM were honeybees early in the season which are considered to contribute to the pollination of *L. sativus* [47], some syrphid flies and a few *Eucera* sp., which are known to prefer plants of the Fabaceae family.

Glebionis coronaria, which provided a long-lasting flowering throughout the season, and *A. graveolens* attracted wild bees. Flowering of these species overlapped with that of the tomato crop and lasted until the fruit-set, providing an attractive habitat for potential pollinators of the crop. *Glebionis* spp. is known to attract wild bees and honeybees (M. Edwards pers. obs.). In our study, *G. coronaria* also attracted syrphids, *Odontomyia* sp. and other flies, as well as Lepidoptera. *Coriandrum sativum*, the second Apiaceae in the WM with intermediate flowering period and duration, also attracted wild bees and fewer honeybees. Studies in other countries have also reported that the main pollinating visitors of *C. sativum* were honeybees and wild bees of the Andrenidae, Halictidae, and Colletidae families (Algeria) [48], or honeybees and *Megachile* ssp. (Pakistan) [45].

Fagopyrum esculentum, which was the main flowering species in the SM, attracted fewer wild bee species (mainly *Andrena* spp. and *Halictus* spp.) but had significantly more visits than the WM, where the main flowering was provided from *C. sativum*, *A. graveolense* and *G. coronaria* in mid-June and *A. graveolense*, *G. coronaria* and *P. echioides* in mid-July. In addition, it attracted syrphids, other flies and Lepidoptera, as also reported by Thapa [49]. Previous studies have reported the capacity of *F. esculentum* to upgrade the floral resources of field margins for the benefit of pollinators and beneficial arthropods [29, 50]. Although the species of pollinating insects visiting the flowers of *F. esculentum* differ in various parts of the world, in most cases, *A. mellifera* is reported as the main pollinator species [41]. Other wild bees foraging on the *F. esculentum* flowers, include *Bombus* spp. [41,44], *Xylocopa* spp., and Halictidae species, including *Halictus* spp. in Florida [41]. In our study, *F. esculentum* attracted both wild bees and honeybees (mid-July). At that time *P. tanacetifolia* was visited by wild bees. In other studies, *P. tanacetifolia* was visited by both honeybees and wild bees [23], or only honey bees [51]. This discrepancy might be explained by the generally low numbers of honeybees in the area at the time of *P. tanacetifollia* flowering (mid-July). The suitability of *P. tanacetifollia* species as a flower resource to honeybees and wild bees should be re-considered, according to some studies [52].

The NV plots had a plant diversity comparable to the sown mixtures, with species flowering early (*R. rugosum*, *A. altissima*) or later in the season (*S. atropurpurea*, *P. echioides*, *D. carota* and *C. arvensis*). However, the overall flowering provision was low and was reflected on the small number of the attracted species and visits of wild bees (*Andrena* spp., *Lasioglossum* spp.). An exception was for *R. rugosum*, which was foraged mainly by honeybees early in the season and a smaller number of wild bees later. *Scabiosa* spp. has been reported to attract wild bees and Lepidoptera, while *C. arvensis* can attract *Lasioglossum* sp. [23]. Nevertheless, these plant species should be managed with caution in

a conservation plan that relies on natural vegetation in the field margins, since *C. arvensis* is a noxious weed [53] and *R. rugosum* has an invasive habit [54]. *Galega officinalis* that emerged in the surrounding area attracted *Bombus* sp., albeit later in the flowering of tomato. In a previous study, *G. officinalis* was among the main plant species that provided floral resources to *Bombus* sp. [55]. Since *Bombus* sp. are among the main pollinating insects for field-grown tomato, the presence of *Galega* sp. in the field margins of this crop is favorable.

In our study, the presence of pollinators on tomato flowers was scarce during full flowering of the crop, regardless of the distance between the tomato rows and the sown mixture in mid-June, coinciding with the end of the sown field margins and the increasing flowering and the peak of *F. esculentum* in the summer mixture. Hence, flowers attractive to Hymenoptera pollinators at the sown margins did not affect visits of pollinators on tomato flowers and subsequently they neither distracted (no effect at the neighboring rows) nor increased pollinators in the tomato field.

The WM attracted an arthropod community (phytophagous insects, natural enemies) of greater diversity in comparison to the SM and the NV sites. It attracted more parasitoid families, with a larger number of individuals throughout the season, although the family sizes fluctuated with time. Overall, the flower cover diversity had only a weak correlation with the beneficial arthropod abundance but not their diversity. This was probably due to the large variability between the various field margin types (sown or with natural vegetation) which may have hindered the evidence of the positive relationship between beneficial arthropods and flowering plant diversity, confirmed in other studies [56].

The WM plots maintained parasitic wasps from 16 families, with Scelionidae and Eulophidae being the most abundant families in both the sown and natural vegetation margins, followed by Eurytomidae, Braconidae, and Ichneumonidae, depending also on time. Tetracambidae was found only in NV1 at the beginning of the season in May. Aphelinidae, including aphid parasitoids (*Aphelinus* spp.) and whitefly parasitoids (e.g., *Encarsia formosa*), was recorded mostly in NV2 in June at the end of flowering of *R. rugosum* and during the flowering of *C. intybus*. Trichogrammatidae was recorded only in NV2 in late-June, when scarce flowering from *C. intybus* and *C. arvensis* was present. This important parasitoid taxa includes species which attack major tomato pests, such as *H. armigera* and the South America tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) [57]. Ceraphronidae were mostly found both in the WM and NV in late June and Encyrtidae even later in mid-July. The two natural vegetation sites had similar overall flower cover, but different plant species present at the end of May when main flowering in NV1 was from *A. altissima* and *C. arvensis* and in NV2 from *R. rugosum*. The flowering of the SM, although practically coming from one species, *F. esculentum*, attracted parasitoids of 5–8 families. These were the same with the predominant and most abundant ones of the WM, at that time of the season. These results agree with Campbell et al. [41] who reported that 62 insect species from 16 families were flower visitors in *F. esculentum* fields, including parasitoids, predators, and pollinators, with the most common ones being parasitoid wasps of various arthropod pests; based on this evidence, they suggested the possible use of *F. esculentum* as a cover crop to enhance biological control of various pest arthropods within cropping systems or to augment local pollinator populations. Moreover, [51] reported other insect groups (e.g., Syrphidae, Sphecidae, Crabronidae, Vespidae, and Scoliidae) as common visitors of *F. esculentum*.

The beneficial effect of enhancing crop fields with nectar-producing flowering plants on parasitism rates and biological control of insect pests has been reported by many researchers [58–60]. Under the scope of functional biodiversity, the potential of the sown mixtures to attract natural enemies for the suppression of the crop pests has been acknowledged in several studies, many of them summarized by Balzan et al. [28]. Our results showed that the floral nectar resources provided in the flower margins, especially early in the season, can serve as a reservoir of parasitoids which would contribute to biological control against several pests that can attack tomato.

The most abundant parasitoid family in both mixtures and natural vegetation, Scelionidae, includes egg parasitoids which have an efficient role in the biological control of pentatomids agricultural pests (Hemiptera: Pentatomidae) [61]. The flowers with narrow and tubular corollas with nectaries at the base such as the heads of the Asteraceae, which were the longest lasting flowering (*G. coronaria* in the WM, from early-May to mid-July) can provide nectar to parasitoids of Braconidae [62]. Both families include parasitoid wasps against *H. armigera*, *T. absoluta*, and Diptera leafminers (*Liriomyza* spp.) [63]. Among the numerous reviewed parasitoid species and families of *T. absoluta*, *Trichogramma* spp. (Trichogrammatidae) are of economic importance and are commercialized used as biological control agents [64]. It is noteworthy that *Trichogramma* spp. were found only in the NV2 site at the late-June sampling, when its relatively low flowering was provided mainly by *C. intybus* and *C. arvensis*. This was surprising because at that time the SM site was dominated by flowering *F. esculentum*, a known *Trichogramma*-attracting species [65] and also reported to increase the longevity and fecundity of Trichogrammatidae [66]. Romeis et al. [65] suggested that the small size of *Trichogramma* spp. renders them easily affected by turbulence, which affects their active flight more than larger species. This could potentially limit the effectiveness of spatially restricted types of habitat manipulation, such as the establishment of plant mixtures in field margins [67] and could have affected the presence of *Trichogramma* spp. in the sites we studied.

The high presence of Aphelinidae parasitoids on Brassicaceae is probably associated with the presence of host aphids on these plants (*R. rugosum*) [68]. Nevertheless, Pteromalidae and Ichneumonidae, present in the WM early in the season and Pteromalidae in the SM later in the season include several parasitoid species of aphids (Universal Chalcidoidea Database). Balzan et al. [28] highlighted the functional value of Apiaceae (*A. gravelonens*, *C. sativum*, *Foeniculum vulgare* Mill.) for several parasitoid wasps (Chalcidoidea, Ichneumonidae, Braconidae, Platygastroidea), whose abundance did not increase by the inclusion of species from Fabaceae, Asteraceae, Polygonaceae and Brassicaceae as expected because the dish-bowl flowers of Apiaceae are a more accessible sugar resource than the bell-funnel flowers of Fabaceae and the head-brush flowers of Asteraceae [39,40,62]. In our study, the relatively high presence of parasitoids towards the end of the season (mid-July) in the WM, when *C. sativum* flowering was ending, was probably maintained by the late flush of flowering in *G. coronaria* and *A. graveolense*, but also by the flowering of the weed *P. echioides*. According to Jana and Shekhawat [69], *C. sativum* and *A. graveolens* have a remarkable pest control impact when sown together, while *A. graveolens* is a good nectar resource for the egg parasitoid *Edovum plutteri* Grissel (Chalcididae) of the major pest of potato *Leptinotarsa decemlineata* Crawford (Coleoptera: Chrysomelidae) that can also attack tomato.

Both sown mixtures were habitats for spiders and generalist insect predators of the families Anthocoridae, Chrysopidae, Coccinellidae, Miridae and Syrphidae that prey on soft body insects such as aphids, whiteflies and thrips. Therefore, the plant species selected for the WM mixture could serve as banker plants for the control of these pests. Balzan et al. [28] argued that *A. gravelonens* and *C. sativum* can support the predator groups of Coccinelidae and Thomissidae (Araneae). Moreover, despite the presence of herbivores on the plants in the sown mixtures, there was no evidence of an increased infestation on tomato plants by aphids, Lepidoptera pests, or Heteroptera bugs, pointing to any disservices of the sown margins. Foraging of Lepidoptera tomato pests such as *T. absoluta* on nectar resources of the flower habitats is a concern [28], but Balzan and Wäckers [70], investigating the impact of flower strips with similar plant species synthesis, did not record an increase in Lepidoptera-caused crop damage. There was no evidence for the increase of the presence *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae) or *Lygus* spp. in the sown mixtures, as found by Balzan et al. [28]. Similar studies have shown that wildflower strips can function as a trap crop for these pests [29] and less fruit damage was recorded in the crop adjacent to these strips [70].

4. Materials and Methods

4.1. Selection of Plant Species

Two plant mixtures, one with winter (WM) and one with summer species (SM) were composed to provide a diverse and long-lasting flower cover in the tomato field margin (Table 2). There is currently no seed house in Greece that would provide seeds of indigenous plant species. For this reason, most of the plant species were selected among those available in the Greek Gene Bank (GGB) of the Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization—ELGO DEMETER, Greece, that propagated them for the purposes of the project. Using indigenous genetic resources was imperative, to avoid genetic erosion of local populations from foreign seed material and to increase the likelihood of the mixture's successful establishment. The final selection among the available plant species was based on criteria described before [23]. Briefly, the selected plants were dicotyledonous species with annual life cycle, recorded in the area, not listed as noxious weeds, and belong to a diverse range of families. In addition, we consulted literature data and results available from previous experiments on the success of species establishment and their field performance (flowering period and duration, attractiveness to pollinators or beneficial insects, relative vigor) for the final composition of the mixture and the relative analogy of each species in it. Based on the above, the WM included a total of seven plant species belonging to three families, as follows: Apiaceae (*Anethum graveolens* L., *Coriandrum sativum* L.), Asteraceae (*Glebionis coronaria* (L.) Cass. ex Spach, *Matricaria chamomilla* L., *Tagetes patula* L.) and Fabaceae *(Lathyrus sativus* L., *Vicia villosa* Roth.). Seeds of these species were provided by the GGB, except for *G. coronaria* which was collected from a spontaneous population in Attica, Greece. The SM included the indigenous Apiaceae *Petroselinum crispum* (Mill.) Johann Mihály Fuss (Fuss). However, since no other summer species was available at the time in the GGB, the SM included also two non-indigenous species widely regarded as highly attractive to honeybees, *Phacelia tanacetifolia* Benth. (Boraginaceae) and *Fagopyrum esculentum* Moench (Polygonaceae) whose seeds are available in the Greek market.

Table 2. Species selected for the mixtures of winter (WM) or summer (SM) dicotyledonous plants, sown in the margins of a processing tomato crop, and corresponding percentage of seed weight and number in the mixture.

		Seed Weight (%)		**Seeds/Species (%)**	
Family	**Species**	**WM**	**SM**	**WM**	**SM**
Apiaceae	*Anethum graveolens*	1.97		33	
	Coriandrum sativum	4		7	
	Petroselinum crispum *		17		54
Asteraceae	*Glebionis coronaria*	7			
	*Matricaria chamomilla**	0.03		7	
	*Tagetes patula**	5		31	
Fabaceae	*Lathyrus sativus*	80		13	
	Vicia villosa	2		1	
Polygonaceae	*Fagopyrum esculentum*		68		11
Boraginaceae	*Phacelia tanacetifolia*		15		36

* Plant species that did not contribute to the flowering of the mixtures.

4.2. Seed-Rate Calculation

The seed-rate per species in the mixtures was calculated taking into account the target plant density, the required percentage of each species in the mixtures and the estimated plant survival rate in the field, and seed parameters like the thousand grain weight and the germination percentage, following seed germination assays. The seed weight in grams

for each species in a mixture (Ws) was the output of the following equation, modified from [23]:

$$Ws = Ps \times \left(\frac{1}{ESR}\right) \times \left(\frac{1}{Pg}\right) \times Tp \times A \times \frac{TGW}{1000}$$

where: Ps = Target percentage of plants per species, as presented in Table 1, ESR = estimated survival rate of plants, Pg = germination percentage from petri-dish assays, Tp = Total target number of plants/m^2 (set at 100 plants/m^2), A = sown area, and TGW = thousand grain weight (g). The relative proportion of the species in both mixtures is presented in Table 2.

4.3. Experimentation Site

The experimentation site was a 2-ha field in the agricultural area of Orhomenos, Viotia (38.501311, 22.918594), one of the main areas in Greece where processing tomato is cultivated (Figure 5). The mixtures were sown in one of the field margins running parallel to a drainage/irrigation channel. Plot size was 14 m^2 (7 m long × 2 m wide), with ten plots for the WM (total sown area 140 m^2) and three plots for the SM (total sown area 42 m^2). The control plots were selected in two separate sites with natural vegetation (NV1 and NV2) along the uncropped area next to the irrigation channel, to cater for the different arable weed communities present around the field. Each control site had three plots (total area per control site 42 m^2).

Figure 5. Experimentation site (38.501311, 22.918594) in Orhomenos, Viotia, central Greece and layout of the sown mixtures (winter WM and summer SM) and the two natural vegetation sites (NV1 and NV2) in the field margin of a processing tomato field. The red lines indicate the crop rows (1–2, 20–21 and 40–41 twin rows from the sown margin) where the presence of pollinators was recorded to assess for possible distraction effect from the sown flowering plants.

Sowing of the mixtures was performed by hand, on 2 December 2014 (WM) and 7 May 2015 (SM), in separate parts of the field margin (Figure 5). The water collected during the winter and spring rains in the irrigation/drainage channel next to the sown field margin, maintained the soil moisture at adequate levels for seed germination and subsequent plant development, eliminating the need for additional irrigation.

The crop was transplanted on 10 May 2015, in twin row sets (0.45 m between each row in the set, and 1.2 m between twin rows). The processing tomato was the joint-less variety H3402 (Heinz) with mid-maturity and medium plant size, commonly planted in the area. Irrigation, fertilizer and crop protection inputs were applied by the farmer, as required.

4.4. Measurements

Measurements of flowering and attracted insects were performed during the main flowering period of the sown plants (WM from early-May to mid-July and SM at mid-June and mid-July), the control sites (NV1 and NV2 from early-May to mid-July), and the tomato crop (T, at mid and late-June), at the dates listed in Table 3.

Table 3. Measurement dates for (a) flower cover (F), Hymenoptera pollinator visits (H) and attracted beneficial arthropods (B) in the sown margin (with winter mixture WM, or summer mixture SM) and the natural vegetation sites (NV1 and NV2), and (b) for number of flowers and pollinator visits in the processing tomato crop (T).

	6-May	21-May	3-June	17-June	23-June	15-July
WM	F, P	F, P, B	F, P, B	F, P	B	F, P, B
SM				F, P	B	F, P, B
NV1	F, P	F, P, B	F, P, B	F, P	B	F, P, B
NV2	F, P	F, P, B	F, P, B	F, P	B	F, P, B
T					F, P	F, P

The methodology was as described before [23] and adapted for the current study. More specifically, the total plant cover and flower cover (total and per species), were visually estimated and expressed as percentage cover/plot in all plots of the sown mixtures and the two control sites. Plant species were identified in situ or when necessary, in the lab using the botanical identification key Flora Europaea [71].

Hymenoptera pollinator visits on the flowers of the sown margins and the control plots, were recorded with visual observation of landings for 4′/plot between 10:30 and 14:30 h. The observations and corresponding counts refer to the foraging visits of pollinators and not their absolute numbers which could not be accurate due to their high abundance and their mobility from flower to flower during the observation time. The same observer recorded the pollinator visits on the flowers of all treatments throughout the sampling period to eliminate potential bias between different observers. Weather conditions which could affect insect populations and their flying (wind, temperature, cloud cover) were also recorded to ensure that measurements were performed near the ideal conditions of, wind up to 2 Beaufort, temperature in the range of 17–30 °C, and cloud cover of up to 50%. The actual weather conditions for the area before and during the experiment as recorded by a nearby weather station, are presented in Supplementary Figure S3. Measurements were conducted in five of the ten plots of the WM, in three plots of the SM and in three plots for each of the NV1 and NV2 sites.

Hymenoptera pollinator visits were also recorded on the crop flowers with visual observation as before, at three sites based on the distance from the sown field margin, starting from the first two twin crop rows next to the margin and moving infield to the 20th-21st and 40th-41st twin rows (Figure 5). Each sampling site had three replications of approximately 14 m^2 (2 twin rows, 7 m long). The number of crop plants and flowers were also recorded for each replication.

Wild bee specimens that required identification after the visual observation measurements, were captured with a sweeping net and were identified later in the lab. Identification of pollinators to genera was performed at BPI, Greece by Myrto Barda, based on the identification keys by Michener [72,73] and verified by M. Edwards, U.K.

Beneficial arthropods were recorded with suction sampling (1′/plot) using a modified leaf-blower (Echo ES-2400, 24 cm^3, Kioritz Corporation, Tokyo, Japan) adapted as described in Stewart and Wright (1995) (Supplementary Figure S4). Measurements were conducted in the other five plots of the WM, three plots of the SM and three plots for each, NV1 and NV2 sites. The collected arthropod samples were kept in the freezer (−18 °C) and sorted according to family, genus, and species (where possible) under a stereo-microscope. Identification of the parasitoid taxa was performed at Alexandru Ioan Cuza University of Iaşi, Romania. Identification of Pteromalidae species was based on

the identification keys by Graham [74]. Pollinators captured in the suction samples were also identified as mentioned above.

4.5. Statistical Analysis

Flower synthesis of both mixtures and natural vegetation sites differed over time. Therefore, the data on the effect of mixture and sampling date on vegetation and flowering cover, as well as on pollinators, natural enemies, arthropod and parasitoid diversity (Shannon H index) were analyzed using two-way ANOVA ($\alpha = 0.05$). Data on the number of beneficial insects (predators, parasitoids, total) and pollinators were transformed to their natural logarithm, to achieve better fitting to the assumptions of the analyses (homoscedacidity). Data on the flower cover percentage were arcsin transformed. When significant, means were separated using Tukey's HSD test. When ANOVA showed no significant interaction, then comparisons among main effects were made. If ANOVA showed significant interaction, comparisons of the simple effects of each factor at the levels of the other factor were made. The statistical analyses were performed using the statistical package JMP (v7, SAS Institute Inc., Cary, NC, USA).

5. Conclusions

Selected flowering plants in the field margin of a processing tomato crop can attract wild bees and other pollinators, as well as beneficial arthropods and have a positive effect on their population abundance and diversity. Our results provide evidence that field margin management with selected plants from a range of families, including Asteraceae, Fabaceae, and Apiaceae, can benefit field-grown tomato, because it attracted wild bee species (e.g., *Lasioglossum* spp. and *Hylaeus* spp.) that can support the cross-pollination of tomato flowers, as well as the creation of a reservoir of parasitoids and predators that could contribute to the biological control tomato pests. The lack of any evidence for the potential distraction of pollinating insects from the processing tomato crop suggests that field margin management with selected flowering plants can be a sustainable approach in this crop. Future research should examine the possible effect of insect pollination on tomato fruit quality attributes and quantify the potential of the sown margins to support biological control of tomato pests.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10051003/s1: Table S1. Mean percentage of flower cover (±s.e.m.) in the winter mixture (WM), the summer mixture (SM) or natural vegetation NV1 and NV2 sites, at the field margins of a processing tomato crop in five sampling dates (May–July 2015). Table S2. Mean number (±s.e.m.) of Hymenoptera pollinator visits/plot/4′ in the winter mixture (WM), the summer mixture (SM) or natural vegetation NV1 and NV2 sites, at the field margins of a processing tomato crop in five sampling dates (May–July 2015). Table S3. Pollinator genera and associated flowering in the sown mixtures and natural vegetation at the field margins of processing tomato crop. Table S4. Arthropod taxa recorded in 1′ suction samples/plot from the sown winter mixture (WM), summer mixture (SM) or natural vegetation NV1 and NV2 sites, at the field margins of a processing tomato crop in four sampling dates (May–July 2015). Table S5. Mean number (±s.e.m.) of Hymenoptera parasitoids and predators in 1′ suction samples/plot from the winter mixture (WM), the summer mixture (SM) or natural vegetation NV1 and NV2 sites, at the field margins of a processing tomato crop in four sampling dates (May–July 2015). Table S6. Parasitoid taxa (mean number (M) and Relative Abundance %) recorded in 1′ suction samples/m2 from the sown winter mixture (WM), summer mixture (SM) or natural vegetation NV1 and NV2 sites, at the field margins of a processing tomato crop in four sampling dates (May–July 2015). Figure S1. Overview of the sown field margin with the winter mixture (WM), during plant growth and flowering. Figure S2. Overview of the natural vegetation at the two sites (NV1 and NV2), separately and in relation to the sown margin and the crop, at different dates. Figure S3. Climatic conditions (temperature, precipitation, wind) in the experimentation area for the year 2015, during the sampling period. The sampling dates are indicated in the x-axis. Figure S4. Suction sampling device: A modified

leaf-blower (Echo ES-2400) operating in reverse mode (suction) and fitted with a mesh bag to collect the insects.

Author Contributions: Conceptualization, V.K., F.K., L.E. and P.V.M. (contribution in the plant species selection); methodology, V.K., F.K., L.E., S.L. and P.V.M.; plant identification S.L. and V.K.; pollinating insect identification, F.K., M.B. and M.E.; beneficial arthropod identification M.-D.M. and M.S.; data analysis, L.E., V.K. and F.K.; writing—original draft preparation, V.K. and F.K.; writing—review and editing, V.K. and F.K.; visualization, V.K. and F.K.; supervision, V.K. and F.K.; funding acquisition, F.K., V.K., (field and laboratory work), and P.V.M. (propagation of selected plant species). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Syngenta, in the frame of its biodiversity project, Operation Pollinator.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding authors (V.K., F.K.), upon reasonable request.

Acknowledgments: We would like to thank, from D. Nomikos S.A. Telis Kyritsis for his technical assistance and scientific advice and the farmer Andreas Skouras, for providing the field and performing the soil preparation for sowing, and from Syngenta Hellas, Voula Kalliakaki and Fotis Andrinopoulos for the financial and technical support of this project.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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So Much for Glucosinolates: A Generalist Does Survive and Develop on Brassicas, but at What Cost?

Verena Jeschke [1], Jacinta M. Zalucki [2], Bettina Raguschke [1], Jonathan Gershenzon [1], David G. Heckel [1], Myron P. Zalucki [3] and Daniel G. Vassão [1,*]

[1] Max Planck Institute for Chemical Ecology, 07745 Jena, Germany; verena.jeschke@gmail.com (V.J.); raguschke@ice.mpg.de (B.R.); gershenzon@ice.mpg.de (J.G.); heckel@ice.mpg.de (D.G.H.)
[2] Centre for Planetary Health and Food Security, Griffith University, Brisbane 4111, Australia; J.Zalucki@griffith.edu.au
[3] The School of Biological Sciences, The University of Queensland, Brisbane 4072, Australia; m.zalucki@uq.edu.au
* Correspondence: vassao@ice.mpg.de

Abstract: While plants produce complex cocktails of chemical defences with different targets and efficacies, the biochemical effects of phytotoxin ingestion are often poorly understood. Here, we examine the physiological and metabolic effects of the ingestion of glucosinolates (GSLs), the frontline chemical defenses of brassicas (crucifers), on the generalist herbivore *Helicoverpa armigera*. We focus on kale and cabbage, two crops with similar foliar GSL concentrations but strikingly different GSL compositions. We observed that larval growth and development were well correlated with the nutritional properties of the insect diets, with low protein contents appearing to exacerbate the negative effects of GSLs on growth, pupation and adult eclosion, parameters that were all delayed upon exposure to GSLs. The different GSLs were metabolized similarly by the insect, indicating that the costs of detoxification via conjugation to glutathione (GSH) were similar on the two plant diets. Nevertheless, larval GSH contents, as well as some major nutritional markers (larval protein, free amino acids, and fat), were differentially affected by the different GSL profiles in the two crops. Therefore, the interplay between GSL and the nitrogen/sulfur nutritional availability of different brassicas strongly influences the effectiveness of these chemical defenses against this generalist herbivore.

Keywords: plant chemical defenses; Brassicaceae; glucosinolates; isothiocyanates; generalist herbivore; *Helicoverpa armigera*

1. Introduction

Plants of the Brassicaceae constitutively produce glucosinolates (GSLs) as a front line defence against herbivory. Although not toxic per se, when brought together with myrosinase enzymes that are maintained in separate cells/compartments, GSLs are hydrolysed to isothiocyanates (ITCs) and other toxic compounds, detonating the so-called "mustard oil bomb" [1–4]. The release of these toxic compounds upon plant tissue damage caused by chewing is meant to be a very effective defence against generalist herbivores [5–7] and the toxic effects of these compounds have been repeatedly shown in various laboratory assays [8–10].

Nevertheless, some 168 species of generalist or polyphagous Lepidoptera across 15 moth families feed on plants in the Brassicaceae, and some 21 are recorded as pests [11]. This includes some key pest insects such as *Spodoptera exigua*, *S. littoralis*, *Mamestra brassicae*, *Trichoplusia ni* and *Helicoverpa armigera*, that were found to metabolize ingested ITCs via conjugation to glutathione (GSH) followed by excretion, suggesting some level of GSL detoxification can play a role when feeding on GSL-defended hosts [12,13]. We attempted to select for improved performance of *H. armigera*, the cotton bollworm, on

brassica hosts by exposing neonates to cabbage, and rearing survivors on artificial diet for consecutive generations starting in October 2012. Surprisingly, we found very little effect of our selection on various performance assays: early larval weight gain, time to pupation, pupal weight, time in pupal stage and survival when assessed after 2, 21, and 29 generations of selection [11]. Indeed, both selected and unselected lines produced pupal weights that would be acceptable in assays, although not as "good" as on the routinely used and nutrient-rich artificial diet; see [14] for the relevance of artificial diets. The most consistent effect on performance was that deriving from the larval diet, with brassica plants having strong negative effects on insect development compared with an artificial diet, and with insects fed on cabbage performing less well than those feeding on kale [11], regardless of the number of ancestral generations fed on brassica plants.

Here we determine if there is a biochemical cost for *H. armigera* feeding on brassicas (common cabbage and kale) differing markedly in GSL profiles (see below). We assessed the usual performance measures of herbivorous insects on plant material: larval weight gain, time to complete developmental stages, pupal weight and adult emergence. We also assessed the nutritional chemistry of host processing and tolerance by measuring GSH, free protein and amino acid contents of larvae, and adult fat and protein contents. Given the major biochemical mechanisms previously described for dealing with common GSL-derived ITCs [12,15], we expected that active and continuous detoxification of cabbage and kale ITCs with GSH would have direct negative effects on cellular GSH levels, with further indirect consequences for protein and amino acid levels (especially cysteine and methionine) in larvae, as well as on the body composition, such as fat levels, in adults.

2. Results

2.1. Diet and Food Plant Composition

Samples of artificial diet, kale and cabbage were analysed for their glucosinolate content (plant samples), total protein, soluble amino acids and water content to compare overall nutritional and toxin load. Both cabbage (6.21 $\pm$ 2.62 nmol/mg DW, n = 6) and kale (6.47 $\pm$ 3.58 nmol/mg DW, n = 5) have comparable total GSL levels (Welch' s t-test, p = 0.954, df = 7.68) but differ in their composition (Figure 1A; Figure S1), as well as in protein levels (Figure 1B) and total soluble amino acid (Figure 1C). The two dominating GSL in cabbage were 4-pentenyl- (4pent) and 3-methylsulfinylpropyl (3MSOP)- GSL (accounting for 92%) and in kale 2-hydroxy-3-butenyl- (2OH3But) and 4pent-GSL were the most abundant (accounting for 73%, Figure 1A). In both plants, the GSL profile was dominated by aliphatic-derived GSL (95% for cabbage, 84% for kale) with lower contributions from indolic (1% for cabbage, 12% for kale) and benzenic GSLs (4% for cabbage, 3% for kale; Figure S1). Soluble protein content was lower in plant samples (2.0% DW in cabbage and 3.8% DW in kale) compared to the artificial diet (5.3% DW, ANOVA, p = 0.031; Figure 1B). In contrast, soluble free amino acids were significantly higher in cabbage (55.49 nmol/mg DW) and kale (120.71 nmol/mg DW) compared to artificial diet (24.71 nmol/mg DW, ANOVA, p = 0.011; Figure 1C). Relative amounts of individual amino acids also differ between both plant samples (Table S1) and generally it is observed that there is a high variance in the detected amino acids levels across all measured samples. Lastly, all diet samples have comparable water content; 84% for artificial diet (variance = 3%, n = 5), 92% (variance = 1%, n = 8) for cabbage and 91% (variance = 1%, n = 10) for kale; plant diets did not differ, ($p > 0.05$, t-test) but both had higher water content that the artificial diet ($p < 0.05$).

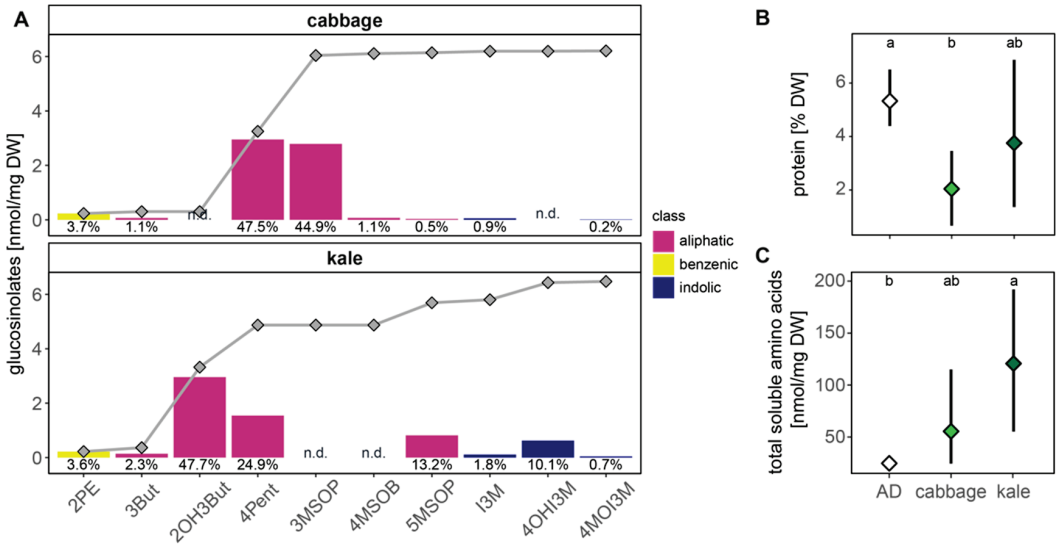

Figure 1. Glucosinolate (**A**), protein (**B**) and soluble amino acid content (**C**) of the food plants and artificial diet (AD). (**A**) Absolute amounts of single glucosinolates [nmol/mg DW] detected in cabbage (n = 6) and kale (n = 5) are plotted as bars, the colour refers to the class of the glucosinolate: benzenic, aliphatic or indolic. The contribution of that structure to the total glucosinolate amount is printed as percentage below the corresponding bar. The dots represent the accumulated means of glucosinolates. (**B**) Soluble protein [% DW] of the diets (p = 0.031) and (**C**) total soluble amino acids [nmol/mg DW] (p = 0.011) plotted as mean ± 95% confidence interval. Tukey letter denoted a statistical difference of 0.05. Mean, standard deviations and statistical details for all analytes are in Table S1. AD = artificial, diet, DW = dry weight, n.d = not detected; glucosinolate side chains: 2PE = 2-phenylethyl, 3But = 3-butenyl, 2OH3But = 2-hydroxy-3-butenyl, 4Pent = 4-pentenyl, 3MSOP = 3-(methylsulfinyl)propyl, 4MSOB = 4-(methylsulfinyl)butyl, 5MSOP = 5-(methylsulfinyl)pentyl, I3M = indol-3-ylmethyl, 4OHI3M = 4-hydroxy-indol-3-ylmethyl, 4MOI3M = 4-methoxyindol-3-ylmethyl.

2.2. Caterpillar Development and Body Chemistry

The effects of different GSL-containing diets on the development of the two *H. armigera* strains were assessed comparing survival, larval weight at 4–5 days after hatching (III instar) and their body chemistry (protein content, GSH level and amino acid levels). We did not find any significant effect of the long-term rearing (selection) of *H. armigera* on cabbage or kale in the larval developmental or chemical responses to the offered diet, thus "strain" was omitted in the analysis and only included as a random factor (nested ANOVA with mixed effect model). Survival of larvae at 4–5 days after hatching (larval stage III) was best on diet, next on kale and worst on cabbage (see [11]). The higher mortalities during the larval stage on cabbage and kale may be due to handling, as their diets were changed every 1–2 days, whereas diet-reared larvae were relatively undisturbed. Mortality was high during the pupal stage for both kale and cabbage reared larvae. Overall survival to the adult stage was 74% on diet, 45% on kale and 28% on cabbage [11].

Larval growth (weight gain) was fastest on artificial diet, and larvae fed with cabbage and kale were differently affected and grew significantly smaller ($p < 0.001$, Figure 2A). Kale-fed caterpillars have similar protein levels as those on control diet; in contrast, cabbage-fed caterpillars, which also showed the strongest growth reduction, had significantly lower protein levels (p = 0.001, Figure 2B). Similarly, the measured decrease of intracellular GSH levels was strongest for cabbage, where levels were reduced to 1% of those of the control group, but kale-fed caterpillars also had significantly lower GSH-levels (14% compared to the control group; $p < 0.001$, Figure 2C). Caterpillar dry weight shows the same pattern as GSH levels (Figure S2), which suggests growth may be coupled via nutrition to GSH metabolism. Both plant-fed caterpillar groups show an increase in total free soluble amino acids ($p < 0.001$, Figure 2D), which suggests an increased body protein degradation. Most individual free amino acids showed increased

levels up to two-fold in plant-fed caterpillars (Figure 2E, Table S2), but there were notable exceptions. Absolute levels of cysteine were lowered in cabbage-fed caterpillar, but with a high variance across the population. Cabbage-fed caterpillars also showed low levels of histidine, but high levels of glutamic acid, proline, and serine. In contrast, histidine levels were elevated in kale-fed caterpillars. Of particular interest are the amino acids building GSH, namely cysteine (and its potential in vivo precursor methionine), glutamic acid and glycine. Relative levels of those amino acids (% of total amino acids) showed interesting patterns (Figure S3). Cysteine contributes the lowest proportion in plant-fed caterpillars, with cabbage-fed caterpillars being most strongly affected ($p < 0.001$). Relative levels of glutamic acid show a similar pattern ($p < 0.001$, while those of glycine are inversely increased ($p = 0.039$). Notably, relative levels of methionine are elevated in plant-fed caterpillars; however, levels are reduced in cabbage-fed caterpillars compared to kale-fed caterpillars ($p = 0.017$). Overall, cabbage-fed caterpillars are more strongly affected in larval fresh and dry weight, protein levels, GSH levels and individual amino acid levels than kale-fed caterpillars.

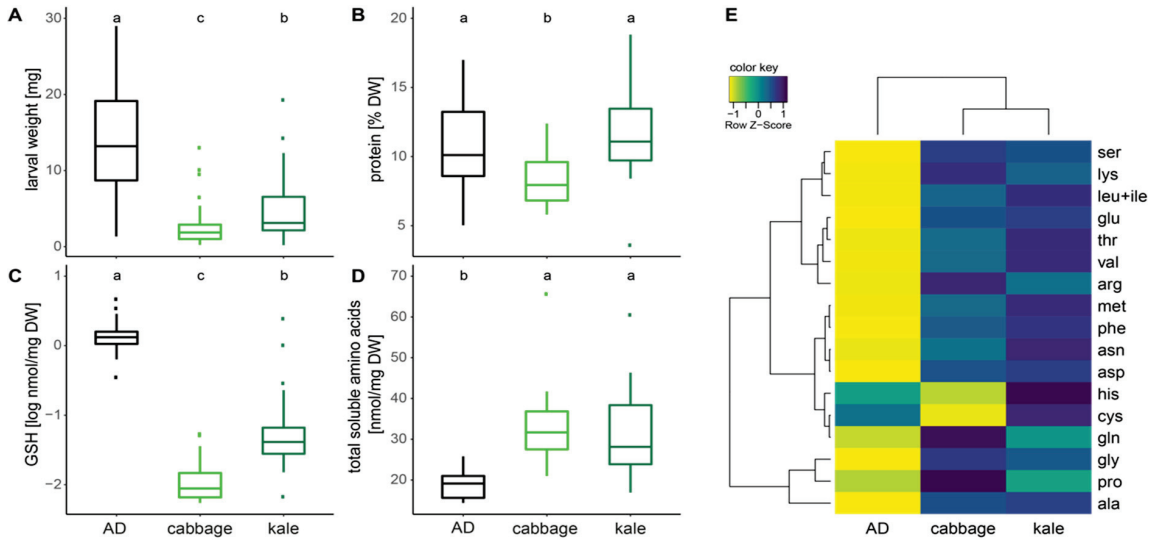

Figure 2. Larval weight and body nutritional composition when fed on artificial diet and food plants with different GSL contents. (**A**) Larval fresh weight at 4–5 days (III instar) in mg ($p < 0.001$, $n = 80$–152); Figure S2 depicts the corresponding values for dry weight. (**B**) Protein in % of dry weight ($p = 0.001$, $n = 20$); (**C**) GSH concentrations in log of nmol/mg dry weight ($p < 0.001$, $n = 20$); (**D**) Total soluble amino acids in nmol/mg dry weight ($p < 0.001$, $n = 20$); (**E**) Heatmap of the mean [nmol/mg DW] of detected soluble amino acids, with yellow to green corresponding to small values and green-blue to dark blue to high values. Data were scaled across rows, comparing amounts of these amino acids between diet treatment groups. Statistical differences for A-D were assessed using a nested ANOVA mixed affect model, letters denote statistical difference at $p = 0.05$ level tested with Tukey post hoc test. Means, standard deviations and statistical details for A-D and individual amino acids (**E**) are in Table S2. AD, artificial diet; DW, dry weight; ■ indicate outliers in the box-and-whiskers plots.

The drastic effects caused by the Brassica diets on larval weight and body chemistry further influence overall caterpillar development time, time in the pupal stage and pupal weight (Figure 3). Caterpillars exposed to GSLs spend more time in the larval stage than caterpillars fed on artificial diet (time point of pupation, Figure 3), with cabbage-fed caterpillars being more affected than kale-fed caterpillars (p(diet) < 0.001, $n = 44$–92). On average, larvae fed artificial diet entered the pupal stage at 12 days after hatching, while the larval stage was prolonged for kale-fed caterpillars for another two days and cabbage-fed caterpillars for another 4–5 days (Figure S4A, Table S3). There is no significant effect on gender alone on the time point of pupation, but a diet-gender interaction; that is, cabbage-fed females enter pupation later than the males, while diet-fed females pupated earlier than the males (p(diet × gender) = 0.003, Figure 3).

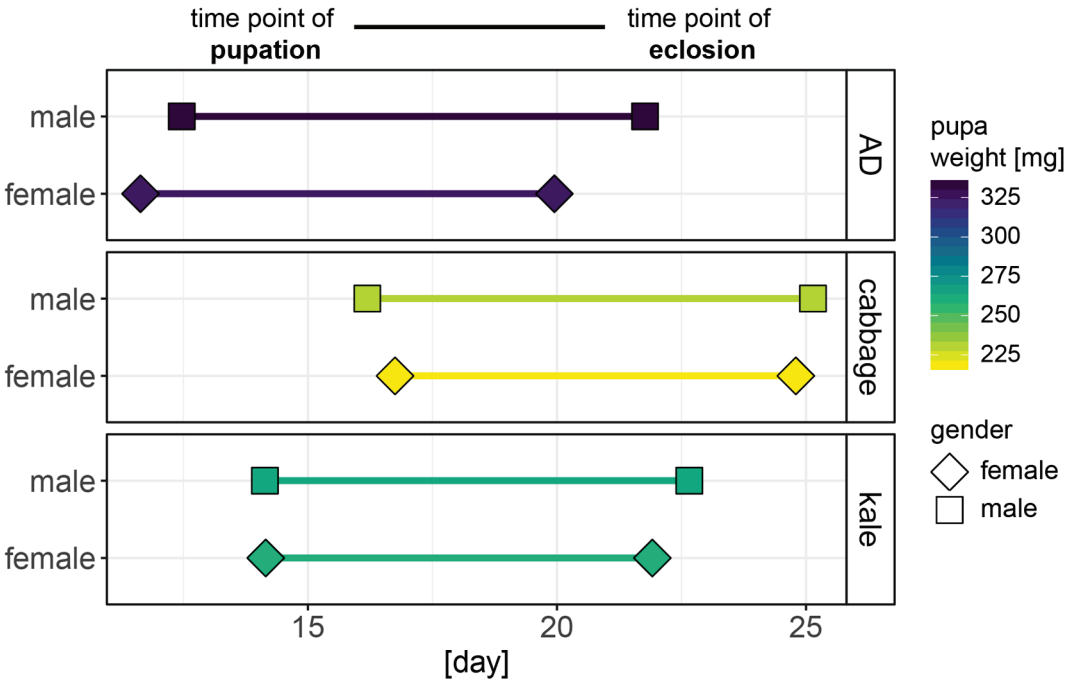

Figure 3. Time to pupation or completion of the larval stage (left data point) and time to adult eclosion (right data point) in days since hatching. The line represents the overall time as a pupa, the colour highlights the pupal weight (taken 24 h after start of pupation). Males are depicted as boxes, females as diamonds, n = 44–92. Means ± SE and statistical information can be found in Figure S4 and Table S3.

Pupal weight showed an inverse pattern to the pupation time points: pupae resulting from larvae fed artificial diet were significantly heavier than those reared on plants (p(diet) < 0.001, Figure 3). Pupae from the cabbage group were 30% lighter, while pupae from the kale group were 20% lighter than those of the control group (Figure S4C, Table S3). There was no significant effect of gender on pupal weight (Table S3), but cabbage-fed females were noticeably lighter than their male counterparts.

Similar to the time point of pupation, the time point of eclosion was significantly delayed for the plant-fed individuals (Figure 3). Interestingly, the duration of the pupal stage was significantly shorter for insects of the cabbage- and kale-treatment groups by 4% and 8%, respectively, compared to the control groups (p(diet) < 0.001, Table S3). Males from the kale group had the strongest reduction of pupal time with a decrease of 0.8 days compared to the control group, while females from the kale group had the overall shortest time of pupation. Overall, females have significantly shorter duration of pupation than males (p(gender) < 0.001). Consequently, females had significantly shorter durations for overall development until eclosion across all diets (Figure 3, p(gender) < 0.001). Despite the quicker pupal development for the plant groups, cabbage- and kale-fed adults emerged significantly later than those of the control groups (p(diet) < 0.001, Figure S4). This resulted in an average shift of eclosion of 4.9 days for cabbage-treated females and 3.3 days for cabbage-treated males, while kale-treated females and males emerged only 2 days and 1 day later compared to the control group (Table S3).

2.3. Adult Body Weight and Composition

Similar to their differences in pupal weights, adults reared on cabbage or kale had lower body weight than those reared on artificial diet (p(diet) < 0.001, Figure 4A), but weights were not affected by gender within a diet type. In contrast, protein levels in male and female adults were differently affected by cabbage or kale feeding (Figure 4B, p(diet) = 0.002, p(gender) = 0.033, p(diet*gender) = 0.008). Females raised on kale had the highest levels of protein, while protein levels were elevated in males raised on cabbage. Body fat mirrored the pattern of fresh adult body weight with adults reared on diet having highest

fat content (28.8%/DW), followed by kale (17.7%/DW) and cabbage (13%/DW; p(diet) < 0.001, Figure 4C). Females from all treatment groups had slightly lower fat levels than the males (p(gender) = 0.041).

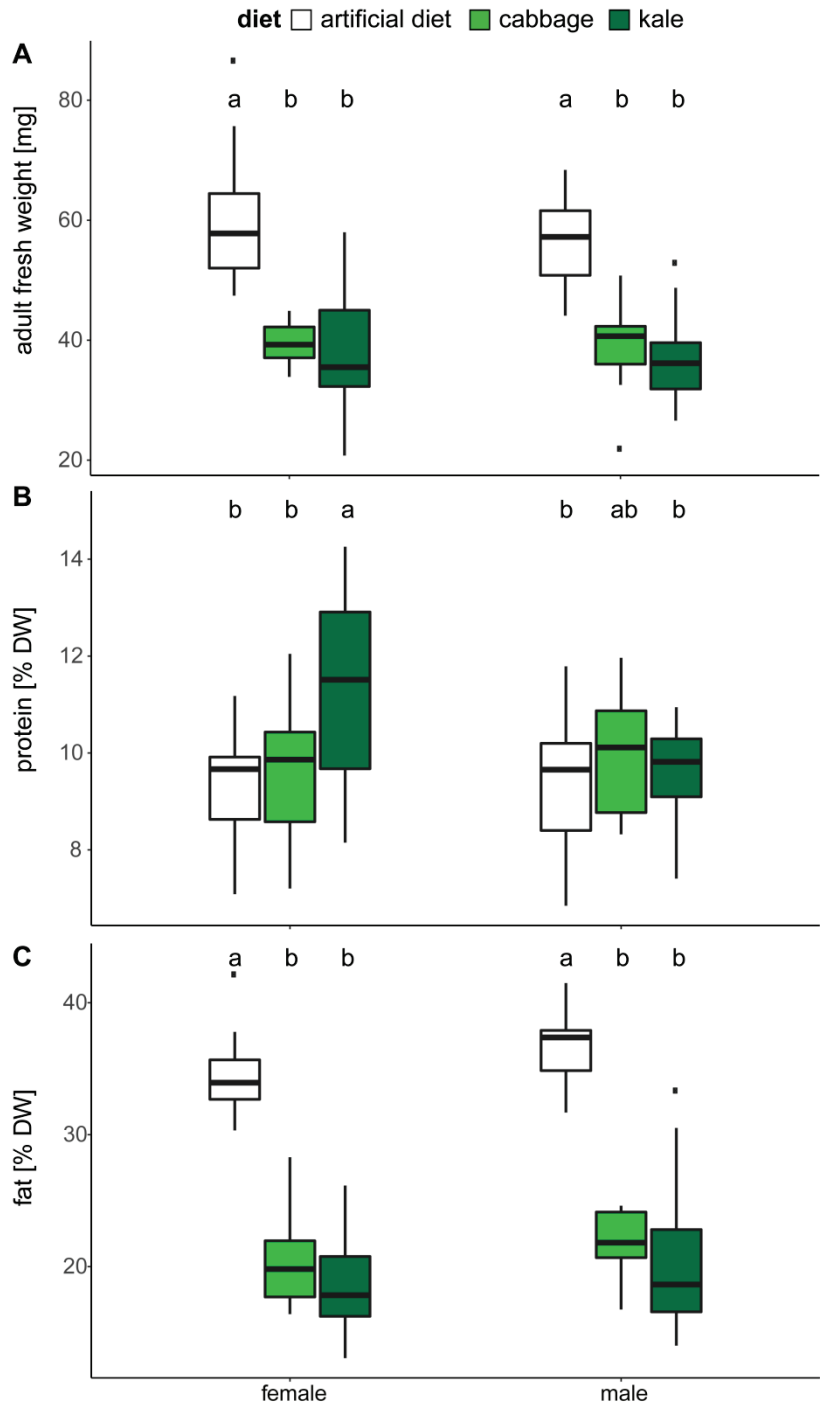

Figure 4. Adult body composition after eclosion. (**A**) Adult fresh weight is strongly affected by the larval diet (p(diet) < 0.001, p(gender) = 0.286, p(diet×gender) = 0.752). (**B**) The amount of protein (per dry weight) is differentially affected by diet for females and males (p(diet) = 0.002, p(gender) = 0.033, p(diet×gender) = 0.008). (**C**) The amount of fat (per dry weight) is mainly affected by larval diet (p(diet) < 0.001, p(gender) = 0.041, p(diet×gender) = 0.885). Mean, standard deviation and statistical details are in Table S4. Statistical differences were assessed using nested ANOVA with mixed effect model, letters denote statistical differences at the p = 0.05 level tested with Tukey posthoc test; ■ indicate outliers in in the box-and-whiskers plots.

2.4. Conjugate Excretion in Faeces

In order to determine the differences in metabolism of glucosinolate-derived ITCs and goitrin, we performed a non-targeted UHPLC-HRMS analysis of frass from *H. armigera* larvae fed on artificial diets containing those compounds. Goitrin is the product of an intramolecular rearrangement (cyclization) of the ITC formed by hydrolysis of 2-hydroxy-3-butenyl glucosinolate, and due to the lack of an exposed ITC group, can be expected to undergo different metabolism than ITCs. This non-targeted analysis indicated that very few products were formed by the caterpillar metabolism, i.e., only a few MS signals were significantly different between extracts of frass from toxin- and control-fed larvae (Figure 5). Most of those products could be readily assigned to putative structures, based on comparison of retention times, molecular masses and fragmentation patterns to product mixtures, as the expected mercapturic acid pathway metabolites. In frass from 3msop-ITC-fed larvae (Figure 5A), signals matching 3msop-ITC and its metabolites 3msop-GSH, 3msop-CG and 3msop-Cys accounted for most of the mass features detected. In frass from 3-butenyl-ITC-fed larvae (Figure 5B), the ITC-Cys conjugate was easily detected, while an additional LC-MS feature corresponded to a product of its intramolecular cyclization, as previously reported for 4msob in slugs [16]. The comparison of extracts from faeces of goitrin- and control-fed larvae (Figure 5C), however, only indicated one product as a potential goitrin product, but its mass and molecular formula did not match any of the expected products of the mercapturic acid pathway. Further analysis of its MS fragmentation strongly suggested that this was a product of an intramolecular cyclization reaction similar to that in slugs [16], but deriving from the goitrin-CysGly conjugate (Figure 5D). A corresponding LC-MS peak was present in a synthetic reaction mixture of goitrin and CysGly. Based on this information, the corresponding cyclic product of the putative goitrin-Cys (Figure 5E) conjugate was detected in the crude reaction mixture of goitrin and Cys, as well as in frass extracts, although its intensity was too low in the latter for it to have been picked by the analysis software. Following the proposed cyclization mechanism (Figure 5F), the corresponding products of goitrin-GSH and goitrin-NAC are less favourably formed, as the cysteine amine is less active as a nucleophile. Accordingly, these compounds were not detected in frass, or in reaction mixes of goitrin with GSH and NAC.

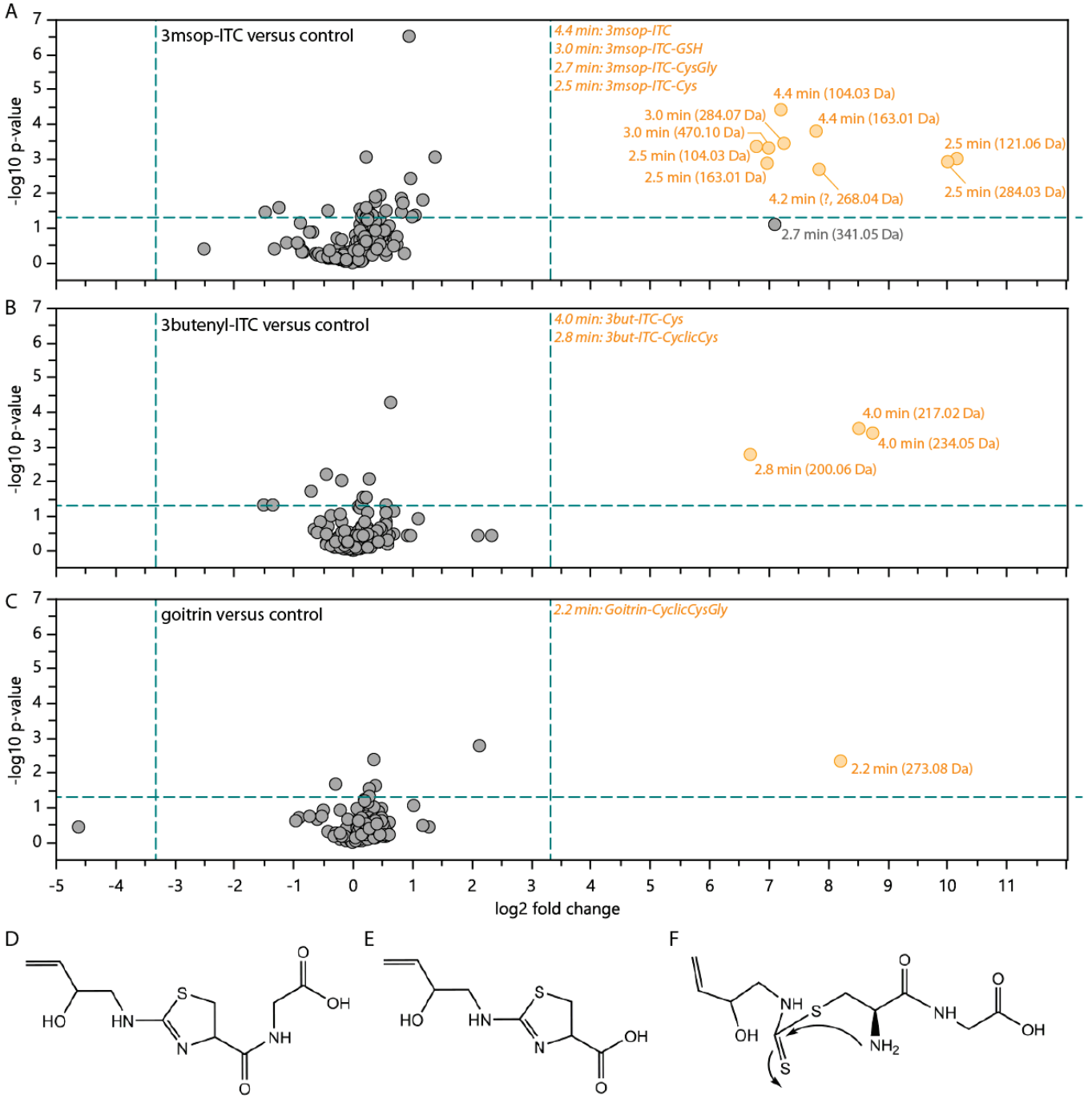

Figure 5. Glucosinolate hydrolysis products are metabolized via the mercapturic acid pathway and intramolecular cyclizations by *H. armigera* larvae. (**A**–**C**) Volcano plots of extracted LC-MS/MS features from non-targeted UHPLC-HRMS analyses indicate that the major metabolites of 3msop-ITC (**A**), 3but-ITC (**B**), and goitrin (**C**) are all formed by conjugation to glutathione (GSH), further hydrolysis of the amino acid constituents of GSH to give CysGly and Cys conjugates, and intramolecular cyclizations to give cyclic conjugates. The proposed goitrin metabolite (**D**) corresponds to the MS feature at 2.2 min in (**C**), while a signal corresponding to the proposed metabolite (**E**) was detectable in manually extracted ion chromatograms from faeces extracts but was too small to be extracted automatically by the software. The proposed formation of these cyclic metabolites is shown in (**F**).

3. Discussion

Plant specialized and defensive compounds are highly variable among plants, and GSL in Brassica plants are no exception. Since these metabolites are key components of several human vegetable crops, they have been extensively measured, and found to vary

greatly in both amount and composition among Brassica crops (e.g., [17]), plant parts (e.g., [18]), among varieties (e.g., [19]) and seasonally (e.g., [20,21]). In the two species studied here, the overall GSL levels were the same but the composition within the group of aliphatic GSLs differed greatly: Cabbage contained mostly 3-methylsulfinylpropyl (3msop)-GSL (absent from kale), and kale 2-hydroxy-3-butenyl- (2OH3But)-GSL (progoitrin, absent from cabbage). These aliphatic GSLs will both generate ITCs upon hydrolysis, with the product of 2OH3But-GSL undergoing further intramolecular rearrangement/cyclization to form goitrin. Post-feeding factors therefore help to modulate toxicity and larval performance.

The proposed insecticide-like toxicity of isothiocyanates (ITC) [22] is believed to derive at least partially from the reaction of the electrophilic ITC group (–N=C=S) with the intracellular tripeptide nucleophile glutathione (GSH), resulting in its depletion and instigating other metabolic consequences [12]. Conjugation with GSH is a common post-ingestion metabolic pathway for mitigating the toxicity of glucosinolate-derived ITCs in generalist lepidopteran herbivores feeding on *Arabidopsis*, including *H. armigera* [13]. The full GSH-ITC conjugate and its downstream CysGly- and Cys conjugates were the major ITC-derived metabolites produced by the generalist *S. littoralis* [12,13].

The balance between conjugation and dissociation reactions is a dynamic process, which can eventually release the individual amino acid constituents of GSH as well as free ITC, which can conjugate with GSH again, leading to depletion of the intracellular GSH pool (Equation 1):

$$\mathrm{ITC} \xrightarrow{+\mathrm{GSH}} \mathrm{ITC-GSH} \xrightarrow{-\mathrm{Glu}} \mathrm{ITC-CysGly} \xrightarrow{-\mathrm{Gly}} \mathrm{ITC-Cys} \quad (1)$$

Equation (1) describes larval detoxification process in gut after ITC ingestion, where ITC-GSH, ITC-CysGly, ITC-Cys represent conjugates of ITCs with glutathione, cysteinylglycine, and cysteine respectively.

To maintain homeostatic levels of free GSH while it is being heavily used in detoxification processes, the insect needs to consistently synthesize more GSH. High protein diets can help replenish the necessary amino acids, particularly cysteine which is the limiting substrate in GSH biosynthesis. A high-protein diet can reduce the influence of potential toxins on the development of *H. armigera* [23]. In this study, we found that kale had a higher protein and soluble free amino acid content than cabbage (Figure 1B,C). Concurrently, cabbage-fed larvae grew more slowly than kale-fed larvae (lower weight at age 4–5 days, Figure 2A), had lower levels of protein (Figure 2B) and GSH (Figure 2C) and took longer to enter pupation and to complete overall development to adulthood (Figure 3). These different physiological effects between cabbage- and kale-fed insects were present despite them having been exposed to similar GSL levels (Figure 1A), and of the major GSL hydrolysis products in each plant (4-pentenyl- and 3-methylsulfinylpropyl-ITCs in cabbage, and goitrin, a rearranged product without the exposed –N=C=S group of ITCs in kale) being presumably metabolized via the same mercapturic acid pathway (Figure 5). This strongly suggests that the nutritional indices of the two host plants, especially their nitrogen and sulphur amino acid contents and bioavailabilities, strongly affect the toxicity of their chemical defences.

While kale-fed larvae contain about 10% of the amounts of GSH of control larvae fed on artificial diet, the GSH levels of cabbage-fed larvae were yet 10-fold lower compared to those of the kale-fed group (Table S2). This suggests a strong depletion of GSH used in combating free ITCs and other oxidative stresses upon feeding on Brassicaceae. Yet, we find significantly increased absolute levels of free glutamic acid (Table S2) suggesting that glutamic acid is not a limiting amino acid; however, relative amounts of Glu (percentage of total amino acids) decrease in plant-fed larvae suggesting an increased usage for GSH synthesis (Figure S3) and/or enlarged free amino acid pools resulting from body protein catabolism. Similar to what had been seen in a previous study [12], cysteine levels were also depleted in *H. armigera* larvae feeding on GSL-containing diets (Figure S3). Due to the

relative toxicity of this sulphur-containing amino acid [24], free cysteine makes up only a small proportion of total free amino acids, about 0.3% for the control larvae and even less in Brassicaceae-fed larvae. It is suggested that in lepidopteran caterpillars more than 20% of the insect´s total cysteine is allocated to GSH [25], making this an invaluable and limiting substance. Hence, the limitations in cysteine availability consequently limit GSH for detoxification; however, critical minimal levels must be maintained for a balanced metabolism to ensure redox homeostasis, on the one hand, and the formation of new proteins for growth or enzymes for essential bioreactions, on the other. That is, lepidopteran larvae can dynamically redirect some of their protein and amino acid resources towards replenishing GSH via an increased availability of its constituent amino acids, especially cysteine. The resulting lower protein levels in larvae exposed to ITCs may be a consequence of an active protein degradation to free up critical amino acids (Figure 2D, Table S2) and energy for detoxification, or from lowered protein synthesis due to limitations in certain amino acids [12]. Here, larvae feeding on cabbage, which produces the aliphatic methylsulfoxide-containing 3msob-GSL (Figure 1), strongly depleted cysteine for use in GSH biosynthesis and resulted in an imbalanced protein metabolism (Figure 2) and thus slower larval growth, in agreement with our previous findings [12]. Interestingly, this effect was also found in parasitoids of *Plutella xylostella* larvae that were raised on GSL-rich *A. thaliana* Col-0 [26]. A recent study, in which *H. armigera* larvae were fed with sinigrin (allyl GSL), confirms that protein metabolism is disturbed in GSL-fed larvae, with upregulations in transcripts related to glutathione and amino acid metabolism [27]. Inversely, adults have elevated protein levels, but significantly lower fat for both genders (Figure 4). This suggests extensive consequences of GSL toxicity on overall resource usage not only during larval development, but also during metamorphosis. The insect fat bodies serve as a central energy storage to power movement and flight and play crucial roles in hormonal regulation and crucially vitellogenesis, an essential step in egg formation and reproduction [28].

Furthermore, we found that individuals raised on GSL-containing plants had significantly longer developmental times for all stages (Figure 3). Again, this effect was stronger for individuals on the cabbage diet (containing the aliphatic 3msop-GSL), and delays in pupations have previously been seen in *S. littoralis* larvae raised on *A. thaliana* Col-0, which contains largely methylsulfoxide-aliphatic GSLs like 4msob and 3msop, among others [29]. Similarly, parasitoids developing on *P. xylostella* larvae that fed on 4msob-ITC-infused leaves suffered from delayed development, lower adult emergence success, and lower body fat content [26] further indicating that GSL-derived compounds affect general animal metabolism, and that these effects are not unique to lepidopteran herbivores.

The prolonged developmental duration and delayed pupation and adult eclosion may have several detrimental implications for the species fitness. A reduced larval growth rate, which has been demonstrated for aliphatic and indolic GSLs affecting different lepidopteran species [7,12,29,30], is detrimental in a natural setting due to longer exposure to predators (slow growth-high mortality hypothesis) and longer times competing for food resources. Another important consequence of late eclosion comes with the overall fitness cost of producing fewer generations per season [31,32]. Interestingly, *S. littoralis* that were raised on cabbage as larvae were shown to avoid Brassicaceae for oviposition [33,34] suggesting behavioural modification to ensure the best chances of survival for the offspring and species.

In conclusion, we show that the lifelong exposure to GSLs and their derived hydrolysis products detrimentally affects insect development and alters body chemistry in the generalist herbivore *H. armigera*. The ingestion of these chemicals led to a depletion of GSH as a consequence of their detoxification via the mercapturic acid pathway, further leading to an imbalanced metabolism of proteins and amino acids in a diet-dependent manner. Somewhat surprisingly, these effects were apparently not ameliorated after continuous rearing of this species on Brassicaceae plants for several generations, suggesting a

lack of plasticity of this ancient and basic metabolic pathway used for detoxification and redox homeostasis. Ultimately, the prolonged developmental times and delayed eclosion caused by GSL-derived toxins, in combination with changes in adult metabolism, can influence the number and health of *H. armigera* larvae and render GSL efficient defensive metabolites against generalist herbivores.

4. Materials and Methods

4.1. Plants

Cabbage (*Brassica oleracea* var capitata, White cabbage "Gloria" (F1 hybrid) from Daehnfeldt Seeds) and kale (*Brassica napus*, Rape broadleaf Essex Salad Sproutin, from B&B World Seeds) were grown in trays (58 cm × 32 cm × 11.5 cm) in a peat-based substrate (Klasmann Kultursubstrat TS1, Geeste-Grob Hesepe) under greenhouse conditions at 21–23 °C, 50–60% RH and 14:10 L:D photoperiod. Each tray contained approximately 60 plants. Multiple trays were established every seven days ensuring similar quality food was available for experiments. Plants were used when they were ca. six weeks old.

4.2. Plant Chemistry and Diet Composition

Six leaves were collected from six week-old plants per species, flash-frozen in liquid nitrogen and lyophilized using a freeze-dryer. Water content in the diet was assessed using gravimetric methods. Dried and ground plant material was analysed for GSL content as described in [29], with *p*-hydroxybenzyl GSL (sinalbin) as an internal quantification standard. The contents of protein und soluble amino acids were determined by extracting 10 mg dried and ground plant material in 100 µL aqueous buffer (Tris, 50 mM, pH 7.5). Protein content was quantified using Bradford assay as described in [12] and soluble amino acid content was quantified via LC-MS/MS as described in [35].

4.3. Insects

The *H. armigera* were from the Toowoomba strain (TWB3) maintained at 28–30 °C on artificial diet purchased from BioServ (Cat. No. F9772, Frenchtown, NJ, USA) and a putative "cabbage feeding" strain we designate as TKF ("Toowoomba Kohlfütterung"). The protocol for maintenance of the insects can be found in [36]. We undertook selection of the TWB3 strain to see if feeding on cabbage in the first instar could select for "better" performance upon GSL exposure. Larvae from isofemale lines were exposed to cabbage leaves in Petri dishes (9 cm diameter) and survivors to the third instar were then reared on artificial diet, mated and the offspring of single pair mating re-exposed to cabbage for each generation starting in October 2012. Subsets of the hatched larvae from each rearing group were used in experiments after 29 generations, in October 2017, as described below.

4.4. Experimental Protocol

We set up single pair crosses within TWB3 and TKF strains and collected eggs daily. Newly hatched larvae (0–8 h old) were introduced to cabbage or kale leaves placed on moist filter paper in Petri dishes, or on artificial diet (9 cm diameter, 10 larvae/dish). All experiments were conducted in a 29 °C environment cabinet, 65% RH, 12:12 L:D (Snijders Scientific, model EB2E).

Larval survival and weight gain were assessed after four to five days; at that temperature larvae were 51–68 degree-days old (about instar III). Larvae used for experiments and sample collections were then individualized into rearing cups (36.9 ml Solo Ultra Clear Portion Containers) with their respective diets and labelled. Subsets of larvae across all families were weighed individually (Mettler XS105 Dual Range balance) before being put into cups. There was sufficient artificial diet in a cup for each larva to feed ad libitum until development was complete. Larvae on kale and cabbage were moved onto fresh leaf material daily till they completed development. The time to pupation was recorded and pupae weighed once the cuticle had hardened (within 24 h). Pupae were checked twice daily, and newly emerged adults sexed.

We collected frass from first instars, late instars and 24 h post molt larvae for each diet treatment and source (TWB3 and TKF, chosen from ca. two larvae per diet per family) for analysis of physiological performance on various diets and targeted metabolite profiling of ITC-derived compounds. These larvae and newly emerged adults (< 24 h old) were collected for profiling of body chemistry and composition. Samples were flash frozen in liquid nitrogen and stored at −80°C until further processing.

4.5. Caterpillar and Adult Body Chemistry

Lyophilized and weighed whole caterpillar and adult samples were ground and extracted with 100 µL Tris buffer (50 mM, pH 7.5) per mg dry weight and analysed for total protein levels using Bradford assay, total fat content using a gravimetric method, and GSH and soluble amino acids using LC-MS/MS as described in Jeschke et al. [12].

4.6. Artificial Diet Feeding and Excretion Assays

Fourth-instar *H. armigera* larvae (n = 15) were fed on an artificial diet mix [36] containing 1 mmol/kg of selected glucosinolate hydrolysis products (3-methylsulfinylpropyl-ITC, 3-butenyl-ITC, or goitrin). Larvae were fed in individual 37-mL plastic cups for 2 days, faeces from groups of three larvae (n = 5) were collected, and 20–40 mg aliquots were extracted with 0. 5 mL methanol:water (1:1) containing 0.5% formic acid (*v/v*) with vigorous shaking. Solid debris were pelleted by centrifugation, and the supernatants were transferred to new vials and analysed by UHPLC-MS.

4.7. Non-Targeted UHPLC-MS Analyses of Larval Frass

Larval faeces extracts were analysed by UHPLC (Thermo Dionex Ultimate 3000) coupled to an HRMS-qTOF MS system (Bruker timsTOF, Bremen, Germany). The UHPLC was equipped with a C18 reversed phase column (Zorbax Eclipse XDB-C18, 1.8 µm, 2.1 mm × 100 mm; Agilent Technologies, Böblingen, Germany) maintained at 25 °C and operated at 0.3 mL/min with a gradient flow of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) with the following profile: 0.5% B from 0–0.5 min, 0.5–80% B from 0.5 to 11 min, 80–100% B from 11–11.1 min and kept at 100% B until 12 min, then re-equilibrated at 0.5% B from 12.1–15 min. HRMS analyses were performed separately at positive and negative ionization modes, with automatic MS2 scans ("autoMS") enabled. The source end plate offset was kept at 500 V and the capillary voltage at 4500 V, with the nebulizer gas at 1.8 bar, dry gas at 10 L/min and a drying temperature of 230 °C. Ion transfer was performed with a funnel 1 RF of 150 Vpp, funnel 2 RF of 200 Vpp, multipole RF of 50 Vpp and a deflection delta of 70 V, with the quadrupole ion energy maintained at 4 eV (low mass 90 m/z), and a collision energy of 7 eV and pre-pulse storage of 5 us. The mass scan range was 50–1500 m/z at an acquisition rate of 12 Hz. Collision energies were stepped in a 50:50 timing between a collision RF of 400 Vpp with transfer time 62.5 us and 800 Vpp/80 us, respectively. Masses were calibrated with m/z of sodium formate adducts injected at the beginning of each chromatographic analysis. Chemical standards of predicted mercapturic acid products of 3msop-ITC, 3-butenyl-ITC and goitrin for chromatographic and MS/MS comparison were prepared synthetically: 5 mM of the GSL hydrolysis products were mixed with 5 mM of glutathione, cysteinylglycine, cysteine or *N*-acetylcysteine in water: ethanol 1:1 at room temperature for 24–48 h, and used directly for HPLC-MS/MS analyses. Data were analysed using the MetaboScape 4.0 software (Bruker, Bremen, Germany), with extraction of features present in at least 3 samples with peak height >5000. Peaks deemed not present in specific samples were assigned an intensity value of 50 counts for fold-change calculations.

4.8. Statistical Analyses

All statistical testing was performed using the statistical software R 3.6.1 (R Development Core Team, http://www.r-project.org, accessed on 7 May 2021). All data were checked for statistical prerequisites such as homogeneity of variances and normality. The

tests used are described in the respective figure and table legends. In short, differences in the diet composition were assessed with ANOVA or Wilcoxon test. Caterpillar and adult chemistry and composition were assessed using a nested ANOVA with mixed effect model (*nlme*-package, [37]) with the strain as the random factor and the diet and gender (for adults) as fixed factors. Since the strain did not have a significant influence on any of the analytes investigated in this study it was not included as a fixed factor for the analysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10050962/s1, Figure S1: Concentrations of glucosinolates from different biosynthetic classes in cabbage and kale, Figure S2: Dry weights of larvae fed on artificial diet (AD), cabbage, or kale, Figure S3: Relative amounts of selected amino acids in bodies of caterpillars fed on artificial diet (AD), cabbage, or kale, Figure S4: Development of larvae fed on artificial diet (AD), cabbage, or kale, (A) duration of the larval stage, (B) duration of the pupal stage, (C) pupal weights, (D) total developmental time until adult eclosion, Table S1: Protein levels, soluble free amino acid composition and glucosinolate compositions of the diets used, Table S2: Larval weights and composition 4–5 days after hatching, Table S3: Duration of insect development, Table S4: Weights and proportions of protein and fat in adult insects.

Author Contributions: Conceptualization, D.G.H., D.G.V., J.G. and M.P.Z.; methodology, V.J., J.M.Z., B.R., D.G.H., M.P.Z. and D.G.V.; formal analysis, V.J., J.M.Z., M.P.Z. and D.G.V.; investigation, V.J., J.M.Z., B.R., D.G.H., M.P.Z. and D.G.V.; resources, J.G., D.G.H., and M.P.Z..; writing—original draft preparation, V.J. and M.P.Z.; writing—review and editing, V.J., D.G.H., D.G.V., J.M.Z. and M.P.Z.; funding acquisition, D.G.H., M.P.Z. and J.G. All authors have read and agreed to the published version of the manuscript.

Funding: MPZ was supported on a Queensland Government International Fellowship in 2012/13. The work was partly supported by ARC DP1095433 and the Max Planck Society.

Data Availability Statement: The data presented in this study are available in the article and in the Supplementary Materials.

Acknowledgments: The work was undertaken while MPZ and JMZ were on leave from UQ and GU. We thank Regina Seibt for maintaining the insect cultures and selection treatment.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

glucosinolate (GSL), isothiocyanate (ITC), glutathione (GSH), 2-phenylethyl (2PE), 3-butenyl (3But), 2-hydroxy-3-butenyl (2OH3but), 4-pentenyl (4Pent), 3-(methylsulfinyl)propyl (3MSOP), 4-(methylsulfin- yl)butyl (4MSOB), 5-(methylsulfinyl)pentyl (5MSOP), indol-3-ylmethyl (I3M), 4-hydroxy-indol-3-ylmethyl (4OHI3M), 4-methoxyindol-3-ylmethyl (4MOI3M).

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A Generalist Feeding on Brassicaceae: It Does Not Get Any Better with Selection

Jacinta M. Zalucki [1], David G. Heckel [2,*], Peng Wang [3], Suyog Kuwar [2,4], Daniel G. Vassão [2], Lynda Perkins [3] and Myron P. Zalucki [3,*]

1 Centre for Planetary Health and Food Security, Griffith University, Brisbane 4011, Australia; j.zalucki@griffith.edu.au
2 Max Planck Institute for Chemical Ecology, 07745 Jena, Germany; suyogkuwar@gmail.com (S.K.); vassao@ice.mpg.de (D.G.V.)
3 School of Biological Sciences, The University of Queensland, Brisbane 4072, Australia; p.wang1@uq.net.au (P.W.); l.perkins@uq.edu.au (L.P.)
4 Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Pune 411007, India
* Correspondence: heckel@ice.mpg.de (D.G.H.); m.zalucki@uq.edu.au (M.P.Z.)

Abstract: Brassicaceae (Cruciferae) are ostensibly defended in part against generalist insect herbivores by toxic isothiocyanates formed when protoxic glucosinolates are hydrolysed. Based on an analysis of published host records, feeding on Brassicas is widespread by both specialist and generalists in the Lepidoptera. The polyphagous noctuid moth *Helicoverpa armigera* is recorded as a pest on some Brassicas and we attempted to improve performance by artificial selection to, in part, determine if this contributes to pest status. Assays on cabbage and kale versus an artificial diet showed no difference in larval growth rate, development times and pupal weights between the parental and the selected strain after 2, 21 and 29 rounds of selection, nor in behaviour assays after 50 generations. There were large differences between the two Brassicas: performance was better on kale than cabbage, although both were comparable to records for other crop hosts, on which the species is a major pest. We discuss what determines "pest" status.

Keywords: glucosinolates; host specialisation; forced selection; performance assays; pest status

1. Introduction

Plants in the Brassicaceae are often portrayed as being a well-defended plant group, with only specialist insects capable of surviving and developing well, while generalists perform less successfully, e.g., [1]. The front-line defences in Brassicales are glucosinolates (GSL), a group of anionic thioglucosides [2,3]. Although GSL are themselves not toxic, when brought together with activating myrosinases that are maintained in separate compartments in plant cells, for example by the actions of a chewing herbivore, they are hydrolysed to form an array of products. These include prominently the toxic isothiocyanates (ITCs), the main components of the so-called mustard oil bomb [4], as well as other hydrolysis products with potential biological activity, namely epithionitriles, simple nitriles and organic thiocyanates, depending on the reaction cofactors and the GSL side chain [5–7]. Due to the reactivities of some of these molecules, especially of ITCs, further intramolecular rearrangements and conjugations to biological nucleophiles are common, leading, for example, to cyclization derivatives, as well as indole carbinols and ascorbigens [5–7]. The release of these toxic compounds upon plant tissue damage caused by chewing is considered to be a very effective defence against generalist herbivores [8–10], and has been repeatedly shown to adversely affect measures of performance of feeding herbivores, for example using *Arabidopsis thaliana* in assays, e.g., [11–14].

Of course, various polyphagous Lepidoptera are recorded to feed on plants in the Brassicaceae. Larvae of several lepidopteran generalist herbivores, including *Spodoptera*

exigua, S. littoralis, Mamestra brassicae, Trichoplusia ni and *Helicoverpa armigera*, for example, are well known "pests" of Brassica vegetable crops. We take more notice of pest species for obvious reasons. Here, we ask how many species in the Lepidoptera other than "pests" are recorded as feeding on Brassicaceae. Is the handful of generalist pests the exception? For one of the pests, the super generalist *Helicoverpa armigera* [15] larvae have been found to produce and excrete glutathione conjugates of ITCs, suggesting that even this species, presumably lacking biochemical adaptations to feeding on Brassicaceae plants, has some level of GSL detoxification, after the de facto chemical defensive ITCs have been formed during ingestion [16].

Although *H. armigera* does not do well on Brassicas, larvae will develop, albeit poorly, on wild type *Arabidopsis,* particularly if allowed to choose feeding sites on whole plants [14]. Cabbage (*B. oleracea* var. *capitata*) is a widely recorded host [15], and larvae of this insect are considered a pest on these crops, particularly in the subcontinent [17–21] and elsewhere [22]. Thakor and Patel [23] readily reared *H. armigera* on cabbage in the laboratory. This suggests that some populations of *H. armigera* do better on Brassica than others, and furthermore, that they might be under selection for better tolerance towards GSL defences, especially where multiple insect generations develop feeding repeatedly on Brassicaceae. That is, it is possible that the pest status of herbivorous species in certain crops may be due to survivors that initially infest a crop and perform better in the next generations, leading to greater pest status. We therefore undertook a laboratory selection experiment to see if the fitness of *H. armigera* on Brassica hosts could be improved, and potentially to help locate the genetic basis for Brassica host use.

Here, we test performance of *H. armigera* on two cultivated Brassicas—common cabbage and kale—that differ markedly in GSL profiles: cabbage with 3-methylsulfinylpropyl (3MSOP)-GSL (absent from kale) and in kale, 2-hydroxy-3-butenyl-(2OH3But) (absent from cabbage) [24]. We undertook selection of the Toowoomba strain (TWB3) of *H. armigera* to see if we could select for "better" performance starting in October 2012 and maintained the trial for 50 generations to November 2019. We designated this "cabbage fed" strain as TKF (Toowoomba "Kohlfütterung"), e.g., refer to 4.3.2.

We undertook three series of rearing assays of both strains: the parental TWB3 and the TKF strains, fed on either artificial diet, cabbage and kale, in January 2013, August 2015 and November 2016, after 2, 21, and 29 rounds of selection, respectively. We assessed several performance measures of herbivorous insects on artificial diet and plant material: larval weight gain, time to complete developmental stages, survival, pupal weight and adult emergence. We undertook simple choice assays to see if feeding preference had changed in first instars (November 2016 and October 2019). We expected TKF to progressively survive and grow better on Brassica plant material and/or show a feeding preference for these plants. Surprisingly, none or only very minor effects were found, but there were major differences in performance measures between the Brassica plants tested that remained relatively consistent in both strains. We discuss the implications of these findings.

2. Results

2.1. Brassica Host Use

Globally, some 317 species of Lepidoptera across 20 families are recorded feeding on Brassicaceae (Cruciferae) in the HOSTS database [25] (Table 1). Some families (Pieridae, Pyralidae and Yponomeutidae) have a high number of crucifer feeding specialists (as expected), but overall, only 21% of moths and butterflies that fed on crucifers were specialists and 26% were oligophagous. What is surprising was the high percentage of species that were polyphagous (56%) recorded on Brassicas with the Noctuidae (97 species), Arctiidae (17) and Pyralidae (17) dominating. Of the polyphagous species, 21 of these (13%) are noted as pests, 11 specialists (13%) are noted as pests and 1 oligophagous species (2%) is noted as a pest.

Table 1. Brassicaceae (Cruciferae) host plant use recorded by lepidopteran families with insect species classified as specialist, oligophagous or polyphagous. The number of pest species, mean number of host plant families (range is min and max), mean % families that are Brassicaceae (Br), mean number of host plant species and mean % of host species that are Brassicaceae (Br). See Supplementary Table S1 for species details.

Family	Specialist	Oligophagous	Polyphagous	Total
Arctiidae		6	17	23
Cosmopterigidae			1	1
Gelechiidae	2	1	1	4
Geometridae	6	7	5	18
Hepialidae			3	3
Lasiocampidae		1		1
Lecithoceridae		1		1
Limacodidae			1	1
Lymantriidae	1		8	9
Lyonetiidae			1	1
Noctuidae	2	25	97	124
Nymphalidae	1	2	1	4
Papilionidae			1	1
Pieridae	34	15		49
Psychidae			2	2
Pyralidae	21	5	17	43
Sphingidae		2	2	4
Tineidae	1	1		2
Tortricidae	1		11	12
Yponomeutidae	14			14
Total	83	66	168	317
Pest Species	11	1	21	
Host Plant Families	1.8 (1–11) *	3.4 (2–8)	16.3 (3–69)	
% Families (Br)	78 (9–100)	35 (13–50)	9 (1–33)	
Host plant species	13 (1–115) *	8.2 (2–54)	57.6 (7–458)	
% species (Br)	93 (56–100)	27 (2–50)	6 (1–23)	

* the outlier is *Plutella xylostella* that is recorded on numerous other families and host species but we retained it as a "specialist" due to its characteristic behaviour towards GSLs and their hydrolysis products [26,27].

2.2. Survival

Early-stage survival in Petri dish assays was high on diet (90–96%) (Figure 1a) and generally lower on cabbage (57–84%) than kale (65–84%). There was no consistent effect of strain and year (Figure 1a). Ignoring the first assessment, survival to adulthood was generally better on diet (62–80%) than plant (42–76%) but declined over time for diet (Figure 1b). The very poor survival to adulthood on plant material in the first assessment (1–5%) was likely an artefact of rearing animals together; even though containers were large and excess food was available, cannibalism was extensive. Therefore, experiments were conducted utilising individualised rearing in subsequent tests.

2.3. Early Larval Weight Gain

Larvae gained the most weight on diet, followed by kale, and grew the slowest on cabbage (Figure 2). Year and food significantly affected the variation in larval weight, but not strain. Larval weight in 2015 was significantly greater than 2013 ($P = 0.042427$), and larval weight was significantly less on cabbage ($P = 0.002044$) and kale ($P = 0.011775$) than on artificial diet. Some 13% of the variation in larval weight left over after fitting fixed effects is due to moth family.

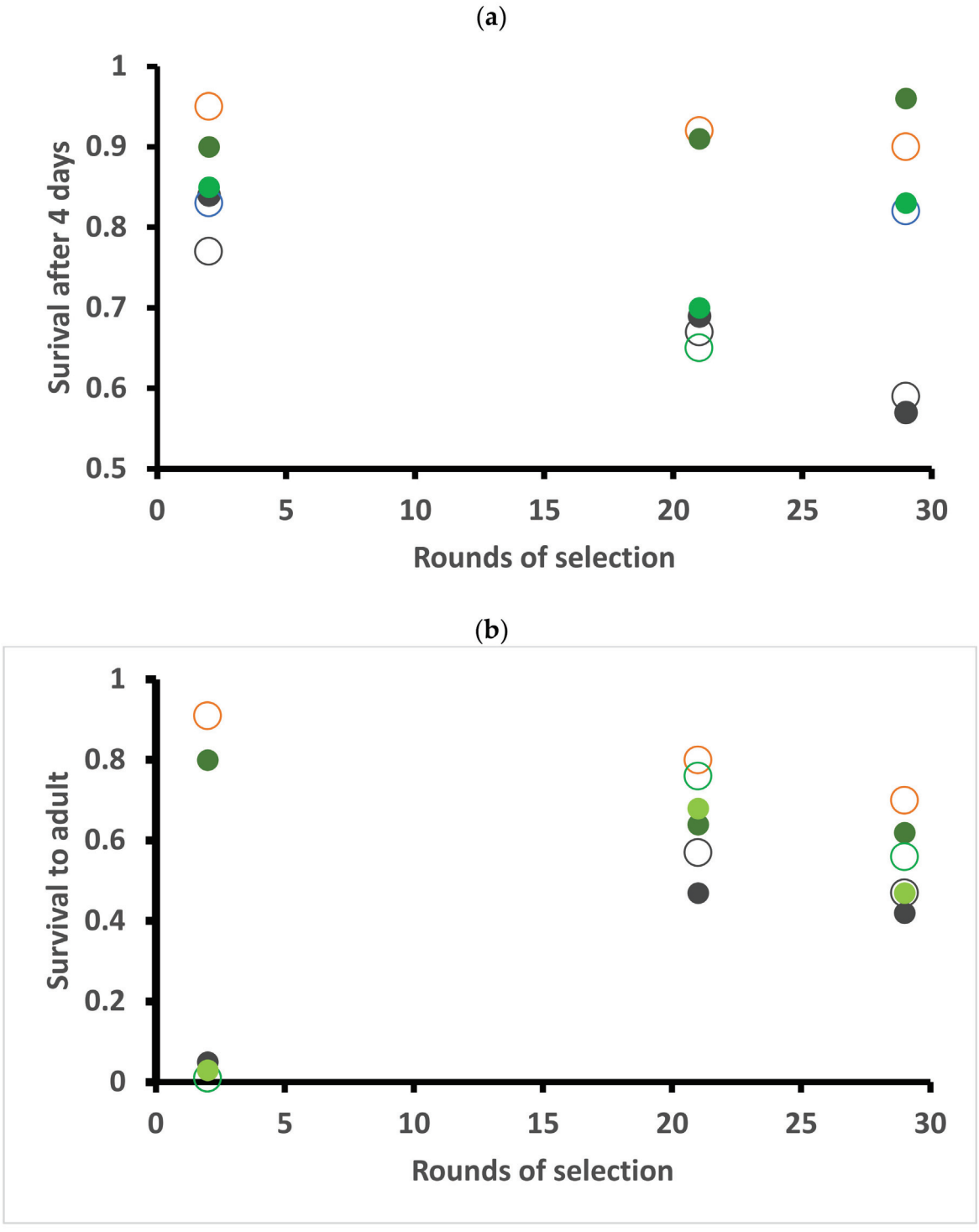

Figure 1. Percentage survival of early instars (**a**) and to the adult stage (**b**) of *Helicoverpa armigera* Toowoomba strain (large open circles) and Brassica-selected strain (solid smaller circles) when reared on artificial diet (brown), cabbage (dark green) or kale (light green), assessed at the indicated rounds of selection (Supplementary Table S2).

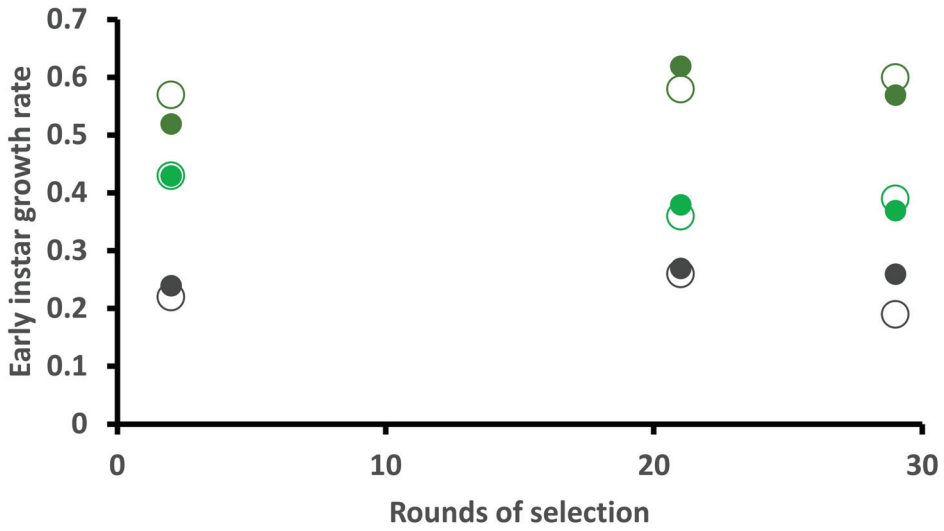

Figure 2. Growth rate (*ln* weight gain/time in days) of early instars of *Helicoverpa armigera* Toowoomba strain (large open circles) and Brassica-selected strain (solid smaller circles) when reared on artificial diet (brown), cabbage (dark green) or kale (light green) assessed at the indicated rounds of selection (Supplementary Table S3).

2.4. Days to Pupation

All data pertaining to pupae were based on data collected after 21 and 29 rounds of selection. Variation in the time taken to pupate is significantly affected by food but not by year, strain or gender. The number of days to pupation was significantly greater for larvae on kale (P = 0.016323) and on cabbage (P = 0.001992) than on diet. Larvae completed development faster on artificial diet (mean ± SD, 12.0 d ± 1.41), followed by kale (14.3 d ± 1.61) and slowest on cabbage (17.4 ± 2.50 d) (Figure 3a). Some 24% of variation in response leftover after fitting fixed effects is due to moth family.

2.5. Pupal Weight

The variance in pupal weight is significantly affected by food, but not by year, strain or gender. Pupal weight was significantly less for larvae raised on kale (P = 0.003708) and cabbage (P = 0.001939) than on diet. Pupae were heaviest on diet (mean ± SD) (324 ± 33 mg), followed by kale (251 ± 38 mg) and cabbage (217 ± 42 mg) (Figure 3b). Only 6% of variation in response leftover after fitting the fixed effects is due to family.

2.6. Time as a Pupa

Variation in time as a pupa (data not shown) is significantly affected by year, food and gender but not by strain. On average, females took a day less to complete the pupal stage (7.8 ± 0.67 d) than males (8.7 ± 0.75 d). Additionally, as with pupal weight, only 6% of variation in response leftover after fitting fixed effects is due to family.

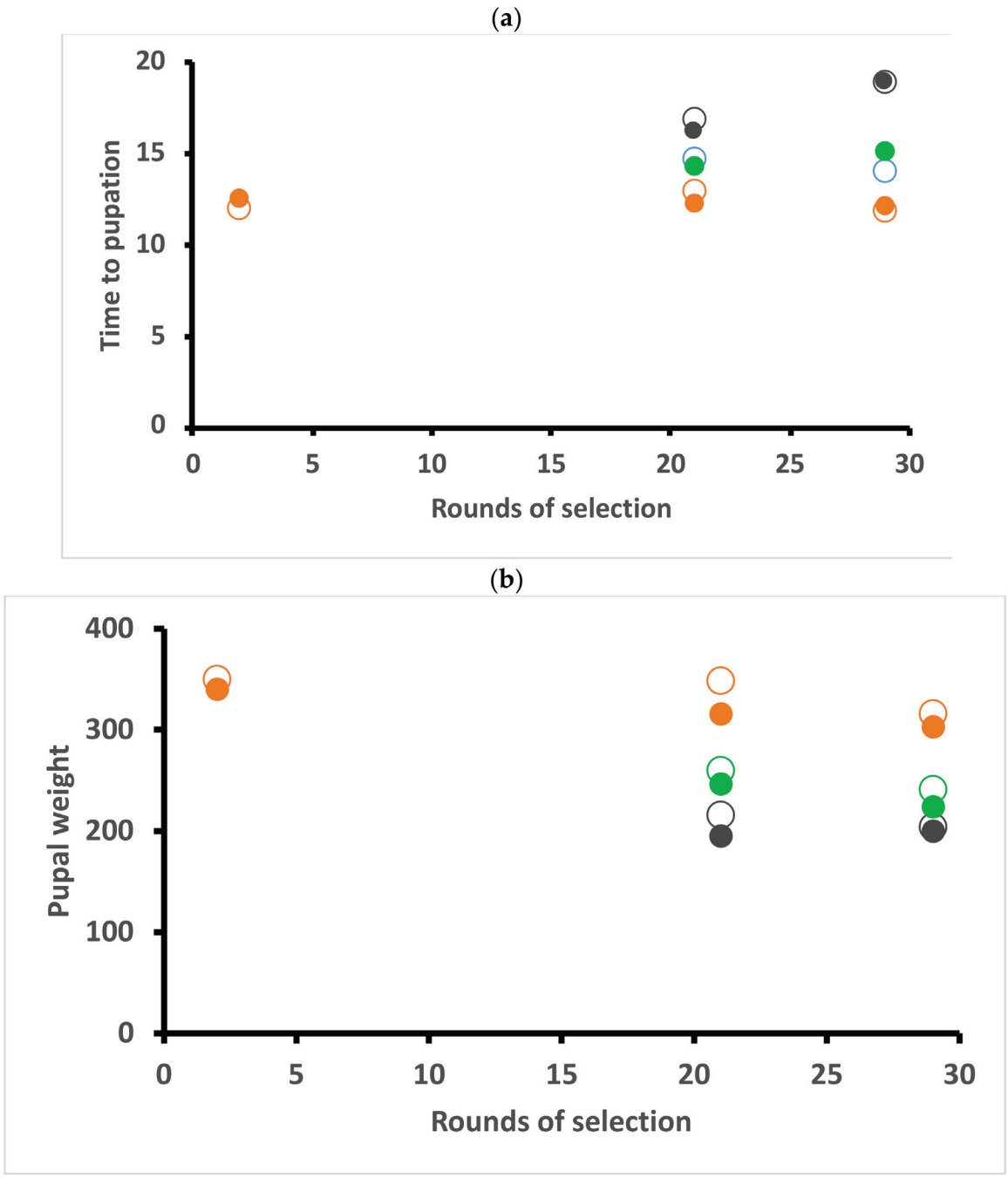

Figure 3. Time to pupation in days (**a**) and pupal weight in mg (**b**) of *Helicoverpa armigera* Toowoomba strain (large open circles) and Brassica-selected strain (solid smaller circles) when reared on artificial diet (brown), cabbage (dark green) or kale (light green) assessed at the indicated rounds of selection. Performance on artificial diet is plotted for the second selection round but were not used in the analysis (Supplementary Table S4).

2.7. Leaving and Choice Assays

In the leaving assay after 29 rounds of selection, a higher proportion of TWB strain neonates abandoned kale (21% ± 4%, SE, n = 6) and cabbage (30% ± 7%) than TKF neonates did—17% (±4%, SE, n = 6) and 15% (±3%), respectively (Supplementary Table S5). However, an analysis on the proportion of larvae that had stayed on the plant disc (1-proportion leaving) at 3 h (logit transformed to give the variable a normal distribution: Shapiro–Wilk normality test, W = 0.9634, P = 0.2734) by two-way ANOVA showed that

neither the strain nor plant (cabbage or kale) significantly affected whether the larvae stayed or left the plant disc at 3 h. When retesting those that left a second time, the rates of leaving for TWB on kale (50% ± 11%) and cabbage (32% ± 11%) were higher than in the first test, and higher than TKF, with 15% (±7%) and 3% (±3) leaving kale and cabbage, respectively. However, the number of larvae being retested was too small for analysis. After 49 generations, in October 2019, we undertook a feeding choice assay on the parental and selected strain. After 48 h, both strains "preferred" the artificial diet, with comparable proportions found on cabbage and kale for each strain (Table 2). A multinomial model fitted to the data showed the best model was Site Found~Plant, i.e., plant (cabbage or kale) is a significant contributing factor (P = 0.0439), but strain was not. We discontinued the experiment and did not run performance assays, as we had expected the TKF strain to differ from the parental strain.

Table 2. Percentage of first instar *Helicoverpa armigera* larvae from selected TKF and the parental TWB strain found on artificial diet, cabbage or kale, and other (agar or Petri dish lid) after 48 h.

Strain	Location Where Larvae Found:			
	Diet	**Cabbage**	**Kale**	**Other**
TKF	51%	26%		23%
	42%		41%	17%
TWB	48%	26%		26%
	44%		34%	22%

2.8. Comparative Performance on Hosts

Larval performance measures on both cabbage and kale were comparable to data published for other *H. armigera* plant hosts (Table 3), including host crop species on which it is a major pest (e.g., Pigeon pea). There is wide variation in measures of so-called performance but as they are all undertaken using plant parts in the laboratory, we make no attempt to analyse these data here.

Table 3. Performance measures (Larval development time in days, Pupal development time in days and Pupal weight in mg) of *Helicoverpa armigera* on a range of host plants under laboratory conditions at a range of temperatures (°C). For multiple studies, we report the range in mean values and just the mean if only one study could be found.

Host	Family	Temperature	Larval Time	Pupal Time	Pupal Weight	Study
Chrysanthemum	Asteraceae	25	20.7		315	5
Sunflower	Asteraceae	27	13.9		200–220	8, 9
Bean	Fabaceae	27	16.62	9.75	257	7
Chickpea	Fabaceae	25	15.6	14.6	260	2
Pigeonpea	Fabaceae	22.5–27	17.5–36.5	13.7–16.2	113–284	11, 12
Cajanus sp	Fabaceae	27	24.8	15.9	207	11
Cajanus sp	Fabaceae	27	35.9	16.8	170	11
Cowpea	Fabaceae	25			210–350	4
Soybean	Fabaceae	25	14.5		300	1, 5
Cotton	Malvaceae	25–27.5	11.4–22.8	10.1–14.2	244–337	1, 5, 6, 7
G. arboretum	Malvaceae	27.5	12.9–17.1	13–14	258–324	6
Okra	Malvaceae	25	13–14.8	14	248–340	1, 3, 5
Corn	Poaceae	25–27	14.5–16.5	9.6–14.2	160–350	3, 4, 7, 10
Chilli Pepper	Solanaceae	27	19.5–21.4	9.79	173–208	7
Eggplant	Solanaceae	25	19.85	14.01	270	3
Pepper	Solanaceae	25	14.1–21.2	14.3	267–290	3,5
Tobacco	Solanaceae	27	15–19.5	10	230–310	7, 8, 9
Tomato	Solanaceae	25–27	13.9–23	9–13.5	167–310	1, 3, 5, 7
Cabbage	Brassicaceae	28–30	17–25	8.6–11	200–224	This study, 13
Kale	Brassicaceae	28–30	14–15.2	8.2	224–241	This study

3. Discussion

Following previous observations that some populations of *H. armigera* perform better than others on the glucosinolate (GSL)-defended plants of the Brassicaceae, we hypothesized that continuous selection might select for better insect tolerance towards GSL defences, with surviving insects and their descendants performing better and leading to greater potential pest status. However, here, we found no effect of selection by exposure of this highly polyphagous pest species to such "sub-optimal" chemically defended hosts on key performance traits across multiple generations. The selected strain (TKF) did not differ from the parental (TWB) strain in survival, early instar growth, time to pupation and time in pupal stage, even after 29 generations of selection. Under laboratory conditions, *H. armigera* survived and grew reasonably well on host plants that produce almost insecticide-like toxins, the pungent GSL-derived isothiocyanates (ITCs) [28]. Larval performance on both cabbage and kale were comparable to data published for other *H. armigera* plant hosts (Table 3), including host crop species on which it is a major pest. The wide variation in measures of so-called performance can be due to many factors [29] but as they are all undertaken using plant parts in the laboratory, they have little bearing on how a population will develop in the field, where natural enemies are present, and when larvae can self-select, to some extent, where and on what they will feed (e.g., [30]). As is the norm, most/all Heliothinae feed on plant reproductive structures [31] and, perhaps not surprisingly, in laboratory assays, larvae grew better on plant reproductive structures (e.g., [32–36]).

Being a pest is not a function of host specialisation. Feeding on brassicas and being a pest on such crops is just as likely in host specialists as it is in generalists (Table 2). We appreciate data for host use have limitations. The Diamondback moth (DBM), *Plutella xylostella*, is widely acknowledged as a host specialist and key pest of brassicas (e.g., [37]), yet in the HOSTS database, it is recorded on 11 plant families—most would say it is polyphagous on this basis—and 103 plant species, but 84% of these are crucifers, and we retained its designation as a specialist on this criterion. The occurrence of DBM on other plant families may be a spill over given its high numbers in brassica crops and at least one of these represented a localised host expansion on to peas [38–40]. Therefore, how does an extreme generalist, *H. armigera*, lacking specific adaptations to brassica hosts, reach pest status on brassica crops?

Pest status is a numbers game and, to a large extent, on the plant part damaged. Generally high pest densities are more problematic but even low numbers of a pest feeding on parts that are to be sold at market may greatly reduce the quality and price. Although brassicas are not preferred oviposition hosts of *H. armigera* in cage choice tests [41,42], if large areas of such non-preferred hosts are available, with little else in the landscape, moths will lay eggs on these plants [43]. Early-stage survival is not high on vegetative plant growth stages, even on plants that *H. armigera* is a key pest, e.g., cotton [44]. Survival improves on flowering plants [34], even if they are genetically modified to express Bt toxins [36,45]. By selecting less toxic plant parts and cannibalism of eggs, survival of early stages can greatly improve [45,46]. If populations in an area are high because of local climate (e.g., [47]) and host plants (e.g., [48,49]), or migration [50], the large number of moths laying eggs on available hosts may increase the rate of cannibalism; first hatching larvae are more likely to eat eggs that are about to hatch [46]. Damage to reproductive structures can greatly diminish the value of the product and, if these structures are available, egg placement and neonate behaviour will take them there [51,52]. Under these conditions, sufficient larvae will survive to older instars. These stages often appear better able to deal with plant toxins [53–55] and of course, pest status increases as larvae consume more, particularly in the last instar [56]. With resistance to insecticides in this species [57] and the sample, spray and pray approach to pest management that effectively removes natural enemies [58] and "pest status" is much more likely. It is these processes that probably lead to local pest status on non-preferred and sub-optimal hosts.

In our experiments, we did find significant year-to-year effects (= when the tests were run), but without a clear link to adaptive effects. Although plants were grown under tight protocols, even subtle differences may be significant, such as "time" of year. Assessments were run in January 2013 (after 2 rounds of selection), August 2015 (21 rounds) and November 2016 (29 round) with the final choice test in October 2019 (49 rounds). Could some of the year effects simply reflect differences between plants due to time of year? The only other effect over time was an apparent decline in performance traits (see Figures 1–3 in results) which may reflect the nature of inbreeding and maintaining isofemale lines [59]. The effect occurred in both strains.

Although the moth "Family" effect was generally small (ca. 6%), it was higher for time to pupation (24%), suggesting some of the effect of host is affected by genetic traits and that there is standing genetic variation for performance on hosts. Perhaps a more targeted selection of such families might be able to shift host plant use?

Behavioural assays showed that cabbage was not a preferred plant in the leaving or choice assay against artificial diet. Larvae were marginally more likely to leave cabbage than kale and they were more likely to be found on kale than cabbage. These larval behaviours were correlated with performance.

The strongest effects were observed in differences between herbivore development on cabbage and kale, especially in comparison to the pinto bean-based artificial diet routinely used for larval rearing. Performance measures were generally better for kale than cabbage. As in most domesticated plants, the chemical toxicity level of our current crucifer crops is low relative to wild types [60]; however, in this case, the two brassicas have very different chemical profiles [24]. The toxicity of isothiocyanates (ITC) is believed to derive from the reaction of the electrophilic ITC group with the tripeptide glutathione (GSH), resulting in its depletion and instigating other metabolic consequences [61], and with amino acid residues of proteins, leading to cleavage of disulfide bonds and secondary/tertiary structural changes [62]. Schramm et al. [16] showed that conjugation with GSH is a common post-ingestion metabolic pathway for mitigating the toxicity of glucosinolate-derived ITCs in generalist lepidopteran herbivores feeding on *Arabidopsis*, including *H. armigera*. The full GSH-ITC conjugate and its downstream CysGly- and Cys conjugates were the major ITC-derived metabolites produced by the generalist *S. littoralis*. Presumably, this ability to conjugate ITC is more easily saturated or not fully activated in neonates and they can survive only lower doses of ITCs. Although plants have a formidable array of defences, insects do successfully feed on plants, albeit poorly in the first instar [63]. Their eavesdropping on metabolic changes within plants and responding via behaviour and induction of their own detoxification systems, enables them to overcome plant defences from time to time [14,30,64,65]. We investigate the physiological effects and costs of feeding on cabbage and kale in a companion paper [24].

4. Materials and Methods

4.1. Brassica Feeding Lepidoptera

We searched HOSTS (a Database of the World's Lepidopteran Hostplants: (https://www.nhm.ac.uk/our-science/data/hostplants/ (accessed on 15 August 2020) [25] for all Brassicaceae (Cruciferae) feeding records). It should be noted that this database uses the old classification, Cruciferae, which is now Brassicaceae [66]. If a lepidopteran was recorded as feeding on a Cruciferae, we downloaded all the host plant records for that species. We take these records at face value. We tallied the number of host families and plant species recorded. We calculated the proportion of families utilised that were Cruciferae and the proportion of host plant species recorded as hosts that were Cruciferae. Each lepidopteran recorded as feeding on Cruciferae was classified as either a specialist (monophagous), generalist (polyphagous) or oligophagous based on the following criteria: the % of host plant families used in the Cruciferae and the % of host plant species used in the Cruciferae:

Host family (x) Host species (y)
Specialist: 9 < x < 100 56 < y < 100
Oligophagous: 13 < x < 50 2 < y < 50
Generalist: 1 < x < 33 1 < y < 23

4.2. Performance on Different Hosts

Published studies of performance of *H. armigera*, perhaps not surprisingly, report a wide variation in fitness traits or performance measures and tend to be fixated on artificial diets [29]. We took a subset of these studies to compare to our findings. The papers had to report data on larvae reared on plant material (not various artificial diets) and at least include pupal weight.

4.3. Assays Assessing the Effects of Selection

4.3.1. Plants

Cabbage (*Brassica oleracea* var capitata, White cabbage "Gloria" (F1 hybrid) from Daehnfeldt Seeds) and kale (*Brassica napus*, Rape broadleaf Essex Salad Sproutin, from B&B World Seeds) were grown in trays (58 × 32 × 11.5 cm) in a peat-based substrate (Klasmann Kultursubstrat TS1, Geeste-Grob Hesepe, Germany) under greenhouse conditions at 21–23 °C, 50–60% RH and 14: 10 L:D photoperiod. Each tray contained approximately 60 plants. Multiple trays were established every 7 d ensuring similar quality food was available for experiments. Plants were used when they were ca. 6 weeks old.

4.3.2. Insects

The *H. armigera* were from the Toowoomba strain (TWB3) maintained on a pinto bean diet at 28–30 °C. The protocol for maintenance of the insects can be found in [67]. We undertook selection of the TWB3 strain to see if we could determine the genetic basis for cabbage feeding and select for "better" performance. Larvae from isofemale lines were exposed to cabbage leaves in Petri dishes (9 cm diameter) and survivors to the third instar were then reared on diet, mated and the offspring of single pair mating re-exposed to cabbage for each generation starting in October 2012 and maintained for 50 generations until November 2019. We designated this putative "cabbage feeding" strain as TKF (Toowoomba Kohlfütterung).

For each performance assessment experiment, we set up single pair crosses and collected eggs daily. Eggs take approximately 40 degree-days above a developmental threshold of 12 °C to complete development [68]. By moving eggs between constant temperatures and manipulating development, we ensured neonate larvae were available for the initiation of each experiment detailed below.

4.3.3. Experimental Protocol

We undertook three series of assessments of both strains: the parental TWB3 and the TKF strain, on artificial diet, cabbage and kale in January 2013, August 2015 and November 2016, effectively after 2, 21, and 29 rounds of selection, respectively. For each assessment, newly hatched larvae (0–12 h old) were introduced to 1–2 cabbage or kale leaves placed on moist filter paper in Petri dishes (9 cm diameter, maximum 10 larvae/dish) as well as 10 neonates onto artificial diet from TKF and TWB crosses (see Table 2 below for a summary of how many Petri dishes and larvae were set up). All experiments were conducted in a 29 °C environment cabinet, 65%RH, 12:12 L:D (Snijders Scientific, model EB2E, Snijders Labs, Tilburg, The Netherlands).

In all experiments, survival and weight of larvae were assessed 3–4 days later; at that temperature, larvae would have been about 51–68 degree-days old (late II or early III instar). In 2013, surviving larvae were transferred to large plastic containers (20 × 30 × 10 cm) lined with moist paper towel and provided with whole stems of food plant material ad libitum or placed in individual cups with artificial diet. In 2015 and 2016, all larvae were individualized into rearing cups after 4 days in Petri dishes with

their respective diets. A subset of larvae was weighed (Mettler XS105 Dual Range balance) before being put into cups to estimate growth rates. There was sufficient artificial diet in a cup for larvae to complete development. Larvae on kale and cabbage were moved onto fresh leaf material daily until they completed development. The time to pupation was recorded and pupae were weighed once cuticle had hardened (within 24 h). Pupae were checked twice daily and newly emerged adults sexed.

The number of successful paired crosses varied amongst experiments and each produced a variable number of viable eggs. Consequently, the disposition of eggs amongst rearing diet treatments was unbalanced (Table 4).

Table 4. *Helicoverpa armigera* single pair matings (moth families) of the Toowoomba strain (TWB3) and cabbage selected strain (TKF) that produced enough fertile eggs to run assays for each assessment (moth family number is arbitrary) in 2013, 2015 and 2016, showing the initial number of first instars larvae exposed to each diet type (AD = artificial diet). Divide the number by 10 to obtain the number of Petri dishes.

2013							
Family Number	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**	**Family Number**	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**
TKF	**AD**	**Cabbage**	**Kale**	**TWB**	**AD**	**Cabbage**	**Kale**
2	20	40	40	12	20	45	40
3	10	10	20	15	10	10	10
4	25	80	85	17	20	60	60
5	10	40	40	19	7	50	40
9	10	20	20				
10	10	20	20				
Total	**85**	**210**	**225**		**57**	**165**	**150**
2015							
Family Number	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**	**Family Number**	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**
TKF	**AD**	**Cabbage**	**Kale**	**TWB**	**AD**	**Cabbage**	**Kale**
11	60	55	55	2	60	90	120
15			15	6	20	15	
19	60	16	60	17	20	7	10
24	100	100	100				
Total	**220**	**171**	**230**		**100**	**112**	**130**
2016							
Family Number	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**	**Family Number**	**Larvae Exposed**	**Larvae Exposed**	**Larvae Exposed**
TKF	**AD**	**Cabbage**	**Kale**	**TWB**	**AD**	**Cabbage**	**Kale**
427	20	30	30	504	20	30	30
429	20	30	30	507	20	30	30
432	20	30	30	510	20	30	25
439	20	30	30	513	20	30	30
445	20	30	30	524	20	30	30
				526	10	30	30
Total	100	150	150	Total	110	180	175

4.4. Behavioural Assays

4.4.1. In 2016

Leaves from kale and cabbage were cut into doughnut-like or annulus shape (the diameter of the outer circle was 5 cm and the inner circle 2.4 cm) and imbedded in agar in the lid of Petri dishes. Twenty newly hatched larvae from each strain were put in the

centre of the inner circle in each treatment. Petri dishes were put on the top of paper cups and the cups were stood in trays with shallow water to record escapes.

After three hours, the larvae on the edge of a Petri dish were counted and removed to a new Petri dish with the same set up. After another three hours, the larvae on the edge of the second Petri dish to which they had been transferred were counted. The assay assessed the likelihood of leaving as the other larvae stayed and were feeding on the leaf material. There were 6 Petri dishes for each strain.

4.4.2. In 2019

Neonate larvae from six TKF crosses and seven TWB crosses were each offered a choice of cabbage vs. artificial diet or kale vs. artificial diet. In most cases, multiple Petri dish were set up with 2 discs of each diet type embedded in agar and 20 neonates were placed in a small well in the centre. After 48 h, the location of all larvae was recorded: on each diet type or elsewhere (agar/dish).

4.5. Statistical Analysis

For all analysis in performance assays, we used a mixed effects GLM. For each response variable (early larval weight gain, time to pupation, pupal weight, and time as a pupa), we fitted a model with Year, Strain, Food and, where relevant, Gender as factors with family as a random variable nested within strain, and interaction between Food and Strain was included in the model. In all cases, data distributions were not Gaussian and the GLMR function in R 4.0.2 [69] was used. Larval weight was expressed as *ln*(weight)/age in days and there were sufficient data for all three years. For pupae-related data, there were too few survivors on plant material in the first year (see results) and we only analysed data for 2015 and 2016.

For the behaviour assay data collected in 2016, essentially a no-choice experiment, the response variable was the proportion of larvae that had stayed on the plant disc at 3 h. A two-way ANOVA was performed after logit transformation of the response variable using R 4.0.2. [69].

For the behavioural data collected in 2019, the response variable was the numbers of larvae found on diet or plant (cabbage or kale) or on the dish, essentially a choice experiment. A multinomial model was fitted to the data using R 4.0.2. [69].

Supplementary Materials: The following are available on-line at https://www.mdpi.com/article/10.3390/plants10050954/s1, Table S1: Crucifer feeding species of Lepidoptera, their family, Number of host families and host plant species recorded in HOSTS, Table S2: Summary of survival data for the selected (TKF) and unselected parental strain (TWB) of *Helicoverpa armigera* after 2, 21 and 29 rounds of selection (Year), Table S3: Summary of early laral weight (mg) for the selected (TKF) and unselected parental strain (TWB) of *Helicoverpa armigera* after 2, 21 and 29 rounds of selection (Year), Table S4: Summary of pupal parameters for the selected (TKF) and unselected parental strain (TWB) of *Helicoverpa armigera* for 2015 & 2016 development assays, Table S5: Summary of the proportion of neonate larvae leaving artificial diet (AD) or Cabbage or Kale for the selected (TKF) and unselected parental strain (TWB) of *Helicoverpa armigera* after 21 rounds of selection.

Author Contributions: Conceptualization, D.G.H., M.P.Z., and J.M.Z.; methodology, J.M.Z., D.G.H., D.G.V., M.P.Z.; software, L.P.; formal analysis, L.P.; investigation, M.P.Z., J.M.Z., S.K., and P.W.; resources, D.G.H., and M.P.Z.; writing—original M.P.Z. and J.M.Z.; writing—review and editing, M.P.Z., J.M.Z., L.P., D.G.V. and D.G.H. All authors have read and agreed to the published version of the manuscript.

Vale Suyog Kuwar: JMZ shared an office with Suyog on one of our visits to the Max Planck Institute for Chemical Ecology. I remember Suyog as a scholar and a gentleman who was actively contributing to this paper until late March 2021. He was a bright young academic that passed away far too early on 2nd April 2021 in India from COVID. We dedicate this publication to his memory.

Funding: This research was funded in part by a Queensland Government International Fellowship to MPZ in 2012/2013. The work was partly supported by ARC DP1095433 and the Max Planck Society.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data reported in this study can be found in the Supplementary Data Files.

Acknowledgments: The work was undertaken while MPZ and JMZ were on leave from UQ and GU. MPZ was supported on a Queensland Government International Fellowship in 2012/13. The work was partly supported by ARC DP1095433 and the Max Planck Society. We thank Regina Seibt for maintaining the insect cultures and selection treatment.

Conflicts of Interest: The authors declare no conflict of interest.

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Endophytic Strain *Bacillus subtilis* 26D Increases Levels of Phytohormones and Repairs Growth of Potato Plants after Colorado Potato Beetle Damage

Antonina Sorokan, Svetlana Veselova, Galina Benkovskaya and Igor Maksimov *

Institute of Biochemistry and Genetics of the Ufa Federal Research Centre, Russian Academy of Sciences, 71 Oktyabrya avenue, Ufa 450054, Russia; fourtyanns@googlemail.com (A.S.); veselova75@rambler.ru (S.V.); bengal2@yandex.ru (G.B.)

* Correspondence: igor.mak2011@yandex.ru

Abstract: Plant damage caused by defoliating insects has a long-term negative effect on plant growth and productivity. Consequently, the restoration of plant growth after exposure to pathogens or pests is the main indicator of the effectiveness of the implemented defense reactions. A short-term *Leptinotarsa decemlineata* Say attack on potato tube-grown plantlets (*Solanum tuberosum* L.) led to a reduction of both the length and mass of the shoots in 9 days. The decrease of the content of phytohormones—indole-3-acetic acid (IAA), abscisic acid (ABA), zeatin and zeatin–riboside—in shoots of damaged potato plants was found. Endophytic strain *Bacillus subtilis* 26D (Cohn) is capable of secreting up to 83.6 ng/mL IAA and up to 150 ng/mL cytokinins into the culture medium. Inoculation of potato plants with cells of the *B. subtilis* 26D increases zeatin–riboside content in shoots and the mass of roots of undamaged plants, but does not influence content of IAA and ABA and growth of shoots. The presence of *B. subtilis* 26D in plant tissues promoted a rapid recovery of the growth rates of shoots, as well as the wet and dry mass of roots of plants after the pest attack, which we associate with the maintenance of a high level of IAA, ABA and cytokinins in their tissues.

Keywords: *Bacillus subtilis*; *Leptinotarsa decemlineata*; *Solanum tuberosum*; abscisic acid; zeatin; zeatin–riboside; indole-3-acetic acid; endophyte

1. Introduction

Damage caused by pathogens and insect pests is among the most important factors that reduce the productivity of agricultural plants. The most effective approaches to reduce such negative impacts are the use of pesticides or the development of resistant varieties by either classical breeding or genetic engineering. Damage caused by defoliating insects reduces the photosynthetically active part of the biomass of plants, and thus plant productivity [1], as the plants must utilize additional required energy to overcome this effect. In the majority of plant species, the activation of defense systems leads to interruption of growth—this effect is commonly known as the growth–defense trade-off [2]. While mechanisms of plant defense against pest insects have been extensively investigated, little attention has been paid to the mechanisms of restoring the growth characteristics of plants after damage caused by defoliators, although this is the most important condition for the formation of stable yields.

The Colorado potato beetle (CPB) *Leptinotarsa decemlineata* Say (Coleoptera, Chrysomelidae) is harmful and adaptive to changing environmental factors, a defoliating insect known as an aggressive invader and for its capacity to rapidly develop resistance to insecticides [1]. All modern approaches for control of CPBs including development of Bt-crops, RNAi-based technologies, and applications of *Bacillus thuringiensis*, etc. are based on the idea of pest eradication [3] but none examine the potential for stimulation of plant growth after insect damage.

The multifunctional effect of Plant Growth Promoting Microorganisms (PGPM) is associated with their ability to increase the availability of minerals for plants, to stimulate plant defense mechanisms to pathogens and phytophagous insects, and to have a direct toxic effect on the pest organism [4]. Some PGPM strains are able to produce phytohormones (auxins, cytokinins), that not only regulate plant growth and development, but also participate in the priming of plant defenses [4,5].

Jasmonic acid (JA) and its active form jasmonoyl-L-isoleucine are currently considered key players in the induction of plant defense reactions and regulation of mechanisms of systemic resistance against defoliating insects [6]. However, implication of classical hormones such as ABA, cytokinins and IAA for reparation of plants damaged by insect pests is not clear [2,7]. The importance of these data for the development of modern integrated protocols that take into account the physiological parameters of plants to increase the efficiency and environmental safety of agrobiocenoses certainly is non-contentious.

The strain *B. subtilis* IB-22 produces zeatin–ribosides [8] and can stimulate more than a 10-fold accumulation of cytokinins in lettuce plants inoculated with this strain. Similarly, the auxin-producing strain *B. methylotrophicus* M4-96 isolated from the roots of maize stimulated the growth of *Arabidopsis thaliana* plants inoculated with this strain [9]. The same compound determined the growth-stimulating effect of the strains *B. amyloliquefaciens* FZB42 [10] and *B. amyloliquefaciens* SQR9 [11]. The ability of the bacterial strain *B. aryabhattai* SRB02 to stimulate the growth of *Glycine max* plants was associated with their production of auxins, zeatin, gibberellins, and ABA. *B. aryabhattai* SRB02-treated plants showed heat-stress tolerance and produced consistent levels of ABA under heat stress [12].

A number of strains of the genus *Bacillus* are able to penetrate into the internal tissues of plants, i.e., exist endophytically. Endophytes can provide benefits to the host plant and under diverse environmental conditions they are capable of interacting with plants more efficiently than rhizospheric PGPM [13]. Enrichment of plants with endophytes can be more advantageous than the cultivation of transgenic plants resistant to pathogens [14]. Implementing artificial plant microbiomes with PGPM that are capable of releasing their metabolites immediately in plant tissues can become a challenging alternative to conventional methods of plant protection and growth regulation [15].

In our previous work we showed that the quantity of *B. subtilis* 26D in shoots of potato plants was about 10^5 cells/mg of fresh weight [15]. Thus, consuming plants containing *B. subtilis* 26D by CPBs resulted in 38.1% larvae death. The presence of endophytic *B. subtilis* 26D strain cells in potato plants enhanced the level of transcripts of jasmonate-biosynthesis genes in plant leaves damaged by *L. decemlineata* [16] and decreased the population density of CPBs under the field conditions [15]. In this context, the question about the influence of *B. subtilis* 26D on growth parameters of potato plants is very important. Is it possible to avoid plant defense/growth trade-offs using endophytic microorganisms?

The aim of this work was to investigate the effect of preliminary inoculation of potato with endophytic bacteria *B. subtilis* 26D and to determine the levels of the phytohormones and growth characteristics of the plants after damage caused by CPBs.

2. Results

2.1. Level of Phytohormones in the Culture Medium of B. subtilis 26D

It was found that *B. subtilis* 26D is capable of secreting 83.6 ng/mL IAA, 103.8 ng/mL zeatin and 46.2 ng/mL zeatin–riboside into the liquid culture medium (Table 1). ABA was absent in the culture medium of *B. subtilis* 26D. Sterile LB medium did not contain phytohormones (Table 1).

2.2. Influence of B. subtilis 26D and CPB on the Growth Characteristics of Potato Plants

Shoot length of undamaged plants treated with bacterial suspension did not exceed the control values. The short-term exposure of water-treated potato plants to CPB feeding

caused a significant (Supplementary Table S1) decrease of growth of their shoots from the 1st to the 9th day after CPB damage (Figure 1A). Importantly, the presence of endophytic *B. subtilis* 26D in tissues of damaged plants led to a reduced negative effect of the CPB. In the latter case, the statistically significant decrease of shoot length as compared to control plants was observed only on the 9th day after exposure of plants to CPB damage (Supplementary Table S1). Two-way ANOVA showed that the length of plant shoots was affected by CPB damage at all time points after damage and their interaction (CPB and *B. subtilis* 26D treatment) just on the 9th day after damage (Supplementary Table S1).

Table 1. Content of phytohormones in the culture medium of *B. subtilis* 26D strain.

	Phytohormones Level, ng/mL of Culture Medium			
	IAA	**ABA**	**Zeatin**	**Zeatin-Riboside**
B. subtilis 26D	83.6 ± 3.9	0	103.8 ± 12.9	46.2 ± 6.0
Sterile LB	0	0	0	0

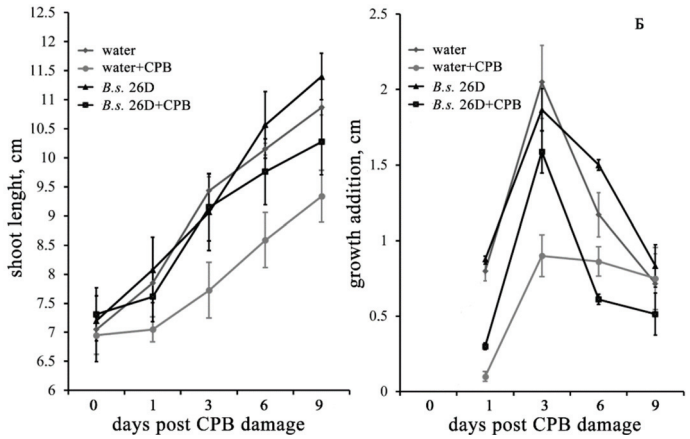

Figure 1. Influence of damage caused by CPB on the length of shoots (**A**) and the growth addition of shoots (**B**) of potato plants treated with water or suspension of *B. subtilis* 26D. The values are means, and the vertical bars represent standard errors. Data analyzed using two-way ANOVA with Tukey's post hoc test. Asterisks indicate means statistically different from the control at $p \leq 0.05$. CPB—plants damaged by Colorado potato beetle.

In both cases, the additive length of shoots of damaged plants significantly decreased on the 1st day after their injury (Supplementary Table S1). However, already on the 3rd day after damage, plants containing endophytic *B. subtilis* 26D showed practically the same growth addition as plants that were not subjected to stress (Figure 1B), while the additional growth of CPB-damaged plants that were untreated with bacterial cells was lower than this parameter of control ones by almost 60%. Later, this parameter of growth of *B. subtilis* 26D-strain treated plants that were exposed to CPB damage decelerated, but the resulting addition of length of shoots of these plants was not significantly different from control scores on the 9th day of the observation. Two-way ANOVA showed that the additional length of plant shoots was affected by CPB damage in the 1st, 3rd and 6th days after damage and interaction of two factors (CPBs and *B. subtilis* 26D treatment) just on the 3rd and 6th days after damage (Supplementary Table S1).

As seen from Figure 2A, the weight of shoots of *B. subtilis* 26D-treated undamaged plants did not show significant differences from control plants, but the fresh weights of their roots exceeded the control values by more than 30%, and the dry weights exceeded those values by 15%. Damage caused by CPBs to water-treated plants resulted in a decrease in both the fresh and dry weights of shoots but did not affect the weight of plant

roots. Both the fresh and dry weights of shoots of damaged plants containing endophytic *B. subtilis* 26D cells also did not differ from the control ones. The fresh weight of the roots was at the level of undamaged plants of this variant, and the dry weight of roots was slightly, but statistically significantly ($p \leq 0.05$) higher than the dry weight of roots of undamaged plants treated with *B. subtilis* 26D cells. According to two-way ANOVA, fresh and dry weights of plant shoots were affected just by CPB damage, but fresh and dry weights of roots were affected by *B. subtilis* 26D and interaction of CPBs and *B. subtilis* 26D treatment (Supplementary Table S2).

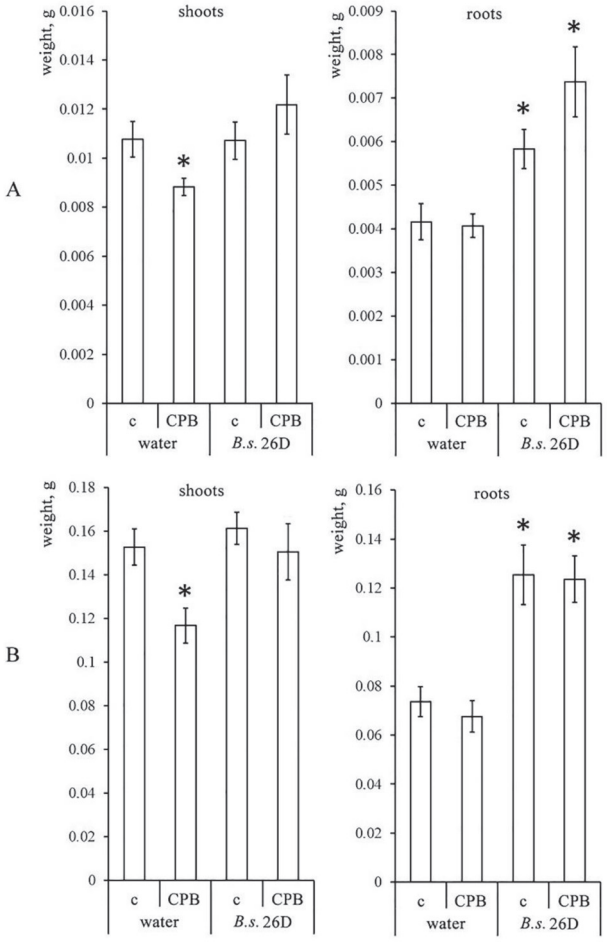

Figure 2. Influence of *B. subtilis* 26D on fresh (**A**) and dry (**B**) weights of shoots and roots of potato plants on the 9th day post damage caused by CPBs. The values are means, and the vertical bars represent standard errors. Data analyzed using two-way ANOVA with Tukey's post hoc test. Asterisks indicate means statistically different from the control at $p \leq 0.05$. c—control plants (water-treated intact plants); CPB—plants damaged by Colorado potato beetle.

2.3. Influence of B. subtilis 26D and CPBs on the Content of Phytohormones in Potato Plants

CPB damage caused a decrease of the level of IAA in potato plants within 3 days after damage (Figure 3A). The level of ABA was reduced on the 1st and 2nd days after exposure to the pest attack, but by the 3rd day after exposure it returned to the control values. In the absence of the stress factor, treatments of plants with *B. subtilis* 26D bacterial cells did not affect the content of IAA and ABA in plants (Supplementary Table S3). After contact with CPBs, levels of both IAA and ABA were consistently higher in *B. subtilis* 26D-containing plants than in control ones. Thus, the ABA level in *B. subtilis* 26D-treated plants after CPB damage was higher than in undamaged plants by more than 40% (Figure 3B) on the 2nd and 3rd days after plants were attacked. Two-way ANOVA demonstrated that the level of IAA was affected by CPB damage at all time points after damage. The

level of ABA was influenced by CPBs on the 1st and 2nd days. The interaction of two factors (CPBs and *B. subtilis* 26D treatment) influenced both these parameters during 3 days after the damage (Supplementary Table S3). *B. subtilis* 26D prevented a decrease of IAA and ABA levels caused by CPB attacks.

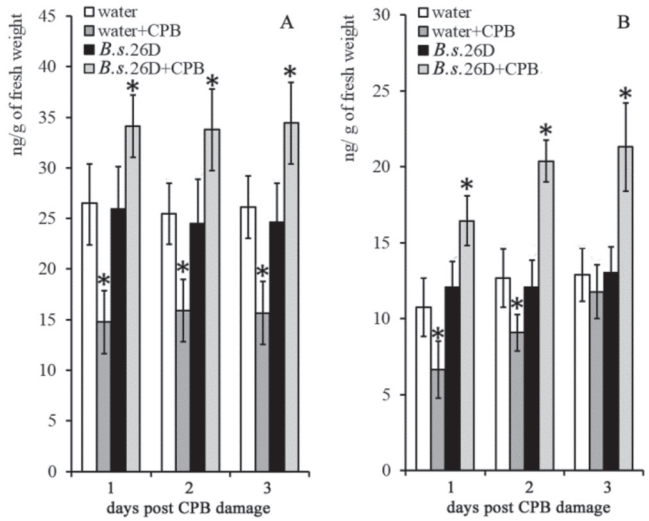

Figure 3. Influence of *B. subtilis* 26D on the level of IAA (**A**) and ABA (**B**) in potato plants on the 1st, 2nd and 3rd day after damage caused by CPBs. The values are means, and the vertical bars represent standard errors. Data analyzed using two-way ANOVA with Tukey's post hoc test. Asterisks indicate means statistically different from the control at $p \leq 0.05$. c—control plants (water-treated intact plants); CPB—plants damaged by Colorado potato beetle.

It was found that on the 1st and 2nd days the level of zeatin (the active form of cytokinins) decreased in water-treated plants damaged by CPBs, after which its content returned to the control level (Figure 4A). In intact plants, treatment with *B. subtilis* 26D had no effect on zeatin content (about 25 ng/g). On the 1st day post damage caused by CPBs, the level of zeatin in *B. subtilis* 26D-treated plants remained equal to the control means and then it increased to 75 ng/g on the 3rd day post damage.

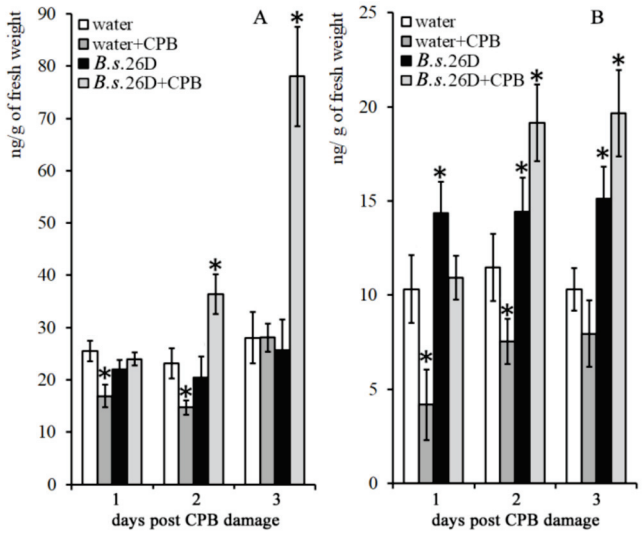

Figure 4. Influence of *B. subtilis* 26D on the level of zeatin (**A**) and zeatin–riboside (**B**) in potato plants on the 1st, 2nd and 3rd day after damage caused by CPBs. The values are means, and the vertical bars represent standard errors. Data analyzed using two-way ANOVA with Tukey's post hoc test. Asterisks indicate means statistically different from the control at $p \leq 0.05$. c—control plants (water-treated intact plants); CPB—plants damaged by Colorado potato beetle.

The level of zeatin–riboside in potato plants that did not contain endophytes was about a third of the control level on the 1st day post damage and then it was about 2/3 of zeatin–riboside content in undamaged plants (Figure 4B). Treatment of plants with *B. subtilis* 26D cells increased the content of zeatin–riboside in intact plants, as evidenced by two-way ANOVA (Supplementary Table S3).

On the 1st day post damage caused by CPBs, the content of zeatin–riboside in *B. subtilis* 26D-treated plants remained equal to the control means and then it increased by a factor of 1.5 on the 2nd and the 3rd day after damage. Thus, *B. subtilis* 26D can stimulate levels of cytokinins in plants after CPB attacks.

Using two-way ANOVA we showed that the level of zeatin and zeatin–riboside was affected by CPBs on the 1st and 2nd days after damage; CPB and *B. subtilis* 26D treatment interaction influenced these parameters on the 2nd and 3rd days after damage (Supplementary Table S3). Treatment of plants with *B. subtilis* 26D influenced the level of zeatin–riboside in all timepoints (Supplementary Table S3).

3. Discussion

It was found that even after a short-time of CPB exposure a serious decrease of growth of potato plants was observed. The study showed that treatment of potato plants with cells of the endophytic strain *B. subtilis* 26D promoted the restoration of plant growth after damage caused by CPBs. Previously, data on the alterations of plant growth by bacteria of the genus *Bacillus* under the influence of biotic and abiotic factors were summarized in the reviews [15,17]. However, investigations of the effect of PGPM on productivity of plants that were damaged by insect pests emphasize the direct insecticidal effect of strains under study [18] or their ability to induce plant resistance [16], but not their effect on the growth of damaged plants.

Treatment of tomato plants with the rhizosphere bacteria *Pseudomonas fluorescens* CHA0 prevented the decrease of plant growth, as well as the fresh and dry weight of shoots and roots, induced by nematodes *Meloidogyne javanica* [19]. However, in the latter case the observed prevention could be due to a decrease of the pest reproduction rate on the roots of treated plants. Saleem et al. [20] showed that *Arabidopsis* plants inoculated with *B. cereus* had higher fitness than uninoculated control ones under the influence of fungus gnats (*Bradysia* spp.).

The secretion of growth-stimulating compounds such as auxins and cytokinins by PGPM is well-known [8,11,21]. However, the question of the source of phytohormones in plants containing endophytes in internal tissues remains open. Endophytic bacteria *B. subtilis* 26D produce a lower volume of cytokinins than super-producers of cytokinins, such as rhizospheric strains *B. subtilis* IB-15 (250 ng/mL) and *B. subtilis* IB-22 (600 ng/mL) [21]. This level is apparently sufficient for the manifestation of growth-compensating activity capable of existing endophytically *B. subtilis* 26D. The endophytic strain *B. subtilis* LK14 isolated from the tissues of *S. lycopersicum* plants produced 57.75 ng IAA/mL of culture medium [22], while bacteria isolated from the rhizosphere produced 100–700 ng IAA/mL of culture medium [23]. However, attention is drawn to the absence of differences in the level of IAA and zeatin in untreated plants and plants treated with bacterial cells without CPB damage. Still the level of zeatin–riboside in *B. subtilis* 26D-treated plants was higher than in control ones, and it is not impossible that cells of bacteria under investigation secreted this transport form of cytokinins in plant tissues. Since bacteria used in the present experiments did not secrete ABA in vitro, changes in ABA level in inoculated plants after CPB damage were not due to the uptake of bacterial ABA, but due to the synthesis by plants itself.

Auxins and cytokinins, considered primarily as phytohormones that regulate plant growth and development, are also involved in the formation of defense reactions against various environmental factors, including attacks of pathogens and pests. It was revealed that after imitation of damage caused by *Manduca sexta* caterpillars the rosette diameter and the number of flowers of *Nicotiana attenuata* plants treated with exogenous IAA increased in comparison with damaged plants treated with water [24]. Yan et al. [25] showed that near the surface of the root transition zone of *Arabidopsis*, the net of auxin flux decreased after a *Plutella xylostella* caterpillar attack. Transcript levels of auxin transporter genes PIN1, PIN2, PIN3, PIN7, and AUX1 were also reduced after the exposure of plants to pest eating. It was assumed that the reduction of growth after insect attack was likely associated with a decrease of auxin transport. Instead, the production of IAA by bacteria is mostly responsible for their root-growth-promoting effects. Thus, *Arabidopsis thaliana* inoculation with IAA-producing bacteria *Azospirillum brasilense* Sp245 increased the number of lateral roots and fresh weight of roots under the normal conditions, and the auxin biosynthesis mutant of this strain did not [26].

In this work, we showed that damage caused by CPBs reduced both plant growth and the level of IAA therein; however, treatment with the suspension of *B. subtilis* 26D cells prevented the decrease of these parameters. Under the influence of the investigated strain, the mass of plant roots increased both under normal conditions and after the pest attack. In contrast to IAA, cytokinins are regarded as an inhibitor of root growth, in particular by promoting cell differentiation in the root apical meristem [27]. But it was found that zeatin–ribosides in bacterial cultural media exist as complexes with high molecular weight polysaccharides, which may prevent their sharp inhibitory effect on root growth [8].

The role of cytokinins is also associated with the availability of macro- and micronutrients and nitrogen, which can have a decisive effect on the growth and development of plants and insects feeding on them [28,29]. Treatment of plants with *B. subtilis* 26D prevented the decrease of the level of zeatin, which was observed after damage by the CPBs in water-treated plants, and contributed to the more than 2-fold increase of this parameter on the 3rd day after contact with the pest. At the same time, the content of zeatin–riboside in intact plants containing cells of bacteria increased. Mechanical injury of *N. attenuata* leaves induced transcriptional changes in many genes of biosynthesis and the signaling pathway of cytokinins, and treatment of plants with zeatin–riboside caused an increase in the content of transcripts of jasmonate-dependent genes, including content of transcripts of gene encoding the trypsin inhibitor, that can repress digestive ferments of insects [28].

Dervinis et al. [29] showed that pretreatment of poplar plants with synthetic cytokinins (6-benzylaminopurine) led to an increase in the content of jasmonic acid and the transcriptional activity of enzymes of its biosynthesis after leaves were exposed to mechanical damage, as well as to a decrease of the mass of gypsy moth *Lymantria dispar* caterpillars receiving 6-benzylaminopurine-treated leaves for food. Thus, the role of cytokinins in plant protection from insects causing extensive mechanical damage may be fundamentally different from the well-known effect of cytokinins produced by gall-forming insects [30]. Thus, cytokinins can promote stomatal opening, stimulate shoot growth and decrease root growth [4]. Arkhipova et al. [8] showed that inoculation with cytokinin-producing bacteria *B. subtilis* IB-22 prevented the decrease of cytokinins level in lettuce plants under the influence of soil drying, and stimulated shoot growth and shoot and root weight. The observed growth promoting effect of these cytokinins on stomatal conductance was attributed to increases in shoot ABA content that *B. subtilis* IB-22 also induced.

In rice plants that overexpressed the protein kinase gene *OsMKK3*, the ABA level increased after damage caused by the brown planthopper *Nilaparvata lugens* [31]. In this case, the survival rate of the pest decreased, as did the height of damaged plants; however, the chlorophyll content in attacked plants did not change, as compared with damaged wild-type plants. In our study, the ABA content in the *B. subtilis* 26D-treated plants increased after the CPB attack, but the length of plants remained at the level of undamaged water-treated plants. The restoration of the plant-growth rate, as well as an increase of the mass of roots in plants containing endophytic *B. subtilis* 26D cells, in which the ABA level was increased, can be indirectly associated with overcoming the stress caused by leaf wilting, which occurs under the defoliation caused by insects [32].

According to the literature, ABA plays an important role in the restoration of growth processes after plant exposure to stress factors. For example, an increase of air temperature led to the termination of leaf elongation in both wild-type barley plants and its ABA-deficient mutant (AZ34), but thereafter the growth rate of leaves of wild-type plants was completely restored [33]. In addition, Arkhipova et al. [7] concluded that *B. subtilis* IB-22 bacteria stimulated the ABA content in shoots of wheat plants and contributed to the increase in the mass of their root system, as in the presented study.

The level of ABA in corn plants (*Zea mays*) increased during the attack of Western corn rootworms (*Diabrotica virgifera virgifera*), but not after mechanical injury caused to the roots [29]. In *Arabidopsis*, after mechanically simulated damage by the desert locust (*Schistocerca gregaria*) the ABA content also did not change [34]. Thus, ABA accumulation can be the common process for plant resistance to insect attacks. In the model plant *Arabidopsis*, the signaling pathway of the defense response induced by JA consists of the MYC and ERF branches, which are regulated by ABA and ethylene, respectively. At the same time, the ethylene-dependent ERF branch is associated with plant protection against necrotrophic pathogens, while the MYC branch, which is additionally regulated by ABA, is associated with wounds or pest damages [35,36].

Arabidopsis mutant *aba2-1* with a low ABA level demonstrated low expression of the marker of MYC-pathway gene *VSP2* and high expression of the marker gene of ERF-pathway PDF1.2 within 30 h after injury caused by caterpillars *Pieris rapae* [35]. Indeed, in our previous work, the mortality of CPB larvae that were directly treated by suspension of *B. subtilis* 26D bacteria exceeded the control values by no more than 15%, while CPB larvae that ate plants containing the endophytic *B. subtilis* 26D strain exceeded these values by no more than 35% at the same time point after exposure [16], which indicated the effectiveness of induced plant defense reactions.

4. Conclusions

We demonstrated the significant decrease of growth of plant shoots that was accompanied by the decrease of IAA, ABA and cytokinins level and, consequently, the importance of the ability of *B. subtilis* 26D to maintain phytohormones levels in plants

and to promote plants' adaptation to CPB-caused damage. The ability of bacteria to increase phytohormones content in potato shoots after the damage caused by defoliating phytophage under investigation was shown for the first time, and this response can promote both the root growth and reparation of shoots growth. We detected that the effect of *B. subtilis* 26D on shoots parameters and the levels of IAA, ABA and zeatin was significant only when plants were affected by CPBs, and therefore the impact of this phytohormone-producing strain cannot deform the phytohormonal status of plants under the normal conditions. We showed that treatment of plants with *B. subtilis* 26D led to an increase of the level of zeatin–riboside in damaged and nondamaged plants, which could be the reason for the promotion of rooting system development. Identification of mechanisms providing a variety of bacterial effects on reparation of plant growth after insect pests' damage and after the influence of other factors should be the subject of further research. However, our results indicate the importance of bacterial regulation of the hormonal status of plants for the activation of plant growth after defoliating insects attacks, as well as the significant contribution of zeatin–riboside accumulation in shoots for the promoting effect of *B. subtilis* 26 D on the rooting system of plants.

5. Materials and Methods

5.1. Plant, Microbe and Insect Material

Plants: sterile *Solanum tuberosum* L. plants cultivar Early Rose obtained by microcloning technology and grown in tubes with Murashige and Skoog medium in a KBW E6 climatic chamber (Binder GmbH, Tuttlingen, Germany) with a 16 h light period at 20–22 °C for 21 days were used.

Bacteria: Gram-positive aerobic *B. subtilis* 26D strain from the collection of the Laboratory of Biochemistry of Plant Immunity of the Institute of Biochemistry and Genetics UFRC [37] were used. Bacteria were grown on liquid Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 20–22 °C using laboratory shakers (120 rpm).

System "Plant + endophyte": 14-day-old plants were inoculated with 5 µL of *B. subtilis* 26D suspension on the stem neighbor of the zone of formation of adventitious roots according to the previously described method [16]. Concentration of bacterial cells was 10^8 cells/mL. Plants were grown in a gnotobiotic system for 7 days, and then the number of endophytic bacteria stood at 10^5 cells/mg of fresh weight. Part of the plants was treated with 5 µL of distilled water (distiller A1110, LLC Liston, Zhukov, Russia) and was used as controls in all experiments.

L. decemlineata: adults were taken from seed potato plants grown on a pesticide-free field of the Chishminsky Plant Breeding Center of the Bashkir Research Institute of Agriculture of UFRC RAS in 2020 (Republic of Bashkortostan, Chishmi, 54°34′49.6″ N 55°25′35.5″ E). Insects were kept under laboratory conditions in pairs in petri dishes. Distilled water in plastic tubes ("Eppendorf", Hamburg, Germany) and fresh potato leaves were replaced as needed.

5.2. Plant Damaging

In order to damage plants treated with water or *B. subtilis* 26D suspension, adult CPBs were placed in test tubes with cultivated plants for 3–5 min, one at a time [16]. During this time, one adult ate about 2–3 mg of leaves of one plant, after which the insect was removed. The level of tissue damage was controlled visually (2–3 mg is the weight of 1/2 leaf) and using CE224-C analytical weight scales (Sartogosm, St. Petersburg, Russia). Plants treated with water and *B. subtilis* 26D, which were not exposed to CPB damage, were used for investigation of all parameters in intact plants. Water-treated intact plants were used as controls in all experiments.

5.3. Plant Growth Parameters

The length of shoots of each plantlet from the base of the stem to the apical bud was measured before the contact with the pest (0 point on graphs), 1, 3, 6 and 9 days after

injury without being removed from the test tube (using aseptic technique). The size of the increment of shoot length, which was grown over consecutive time spans, was calculated for each plant. On the 9th day after exposure to CPBs, damaged and intact plants were removed from test tubes; their roots were washed from the agar medium by washing with running water, and then thoroughly dried on filter paper. Roots and shoots were weighed separately on CE224-C analytical weigh scales (Sartogosm, St. Petersburg, Russia), then the weighed portions were dried twice at 60 °C for 1 h in the SNOL 67/350 dry heat oven (AB Umega, Utena, Lithuania) to measure the dry weight.

5.4. Phytohormones Assay

Bacterial and plant materials processing. Liquid culture medium obtainable by cultivating *B. subtilis* 26D was collected at the late logarithmic growth phase or at the beginning of the stationary phase (on the third day) and centrifuged at 4000× *g* for 20 min in Avanti J-E centrifuge (Beckman Coulter, Bray, OK, USA). The supernatant was analyzed for the content of phytohormones (cytokinins and IAA).

To analyze the levels of cytokinins, IAA, and ABA in shoots (stem with leaves) of test-tube potato plants on the 1st, 2nd and 3rd day after exposure to CPBs, the plant material was homogenized in 80% ethanol (wet weight:volume of ethanol = 1:10) and extracted for 16–20 h at 4 °C. Then the extract was separated by centrifugation at 4000× *g* for 20 min in an Avanti JE centrifuge (Beckman Coulter, Bray, OK, USA) and evaporated to an aqueous residue [37,38].

Cytokinins assay. Cytokinins from 2 mL of the supernatant of the bacterial culture liquid were twice extracted with n-butyl alcohol in a 2:1 ratio (aqueous phase/organic phase) [37]. The extract was evaporated to dryness. Cytokinins from the aqueous residue of plant extract were concentrated by passing through a pre-wetted C18 reverse phase column (Bond-Elut, RP-C18; Varian Ltd., Walton-on-Thames, UK), eluates were evaporated to dryness.

After solvent evaporation, the dry residues in either case were dissolved in 0.02 mL of 80% ethanol and applied to silica gel thin-layer chromatography plates 60 F-254 (Merck, Darmstadt, Germany). Chromatography was carried out in the solvent system butanol: ammonia:water (6:1:2) to separate and assay zeatin–riboside (Rf 0.4–0.5) and zeatin (Rf 0.6–0.7). Zones that contained cytokinins (based on position of standards) were eluted with 0.1 M pH 7.4 phosphate buffer for 16 h, then the silica gel was removed by centrifugation at 10,000× *g* for 10 min in 5415K centrifuge (Eppendorf, Hamburg, Germany). Aliquots of supernatant were added to microplate wells in serial dilutions to assay the cytokinins level [39]. Specificity of antibodies against zeatin–riboside used for ELISA was described previously [40]. The reliability of the hormone immunoassay was confirmed using the dilution test, and results correlated with the results of high performance liquid chromatography (HPLC) in combination with mass spectrometry [8, 40].

IAA and ABA assays. IAA and ABA were extracted from aqueous residues (which were obtained after bacterial and plant materials processing) with diethyl ether as described previously [14]. ABA and IAA were separated with diethyl ether from the aqueous residue, after their dilution with distilled water and acidification with HCl to pH 2.5 (organic phase:aqueous phase being/3:1). Then, hormones were transferred from the organic phase into a solution of $NaHCO_3$ (organic phase: aqueous phase being/1:3), and were re-extracted from the acidified aqueous phase with diethyl ether. At the next stage, the samples were methylated using diazomethane and evaporated to dryness. Dried residues were dissolved in 80% ethanol. An IAA and ABA quantitative assay was performed with ELISA using specific antibodies as described previously [33]. Phytohormones content in bacterial culture medium and plant shoots was calculated per ml culture medium and mg of wet weight, respectively. The sufficiency of hormones purification prior to immunoassay was proved by studying the chromatographic distribution of the immunoreactive material previously [8].

5.5. Statistical Analysis

A total of 30 plants were used in each variant for growth-parameter estimation. For estimation of phytohormones content, six replicates were used in each variant and shoots of four plants were sampled for each repetition. Phytohormone content in the culture medium of *B. subtilis* 26D was investigated in 10 independent flasks.

In order to assess the effect of the treatments on growth parameters and phytohormones levels, 2-way ANOVA was used, setting *B. subtilis* 26D treatment and CPB damage as fixed factors (See Supplementary).

Data showed mean values with standard errors (±SE). Asterisks indicate significant differences among treatments and intact water-treated plants in the same day according to Tukey's HSD multiple range tests at $p \leq 0.05$. Statistica 12.0 (Stat Soft, Tulsa, OK, USA) and Excel 2010 (Microsoft, Redmond, WA, USA) software were used.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10050923/s1 Table S1. Impact of CPB damage and *Bacillus subtilis* 26D treatment on the length of shoots and the rate of growth addition of shoots of potato plants. Data were analyzed using two-way ANOVA with CPB damage and B. subtilis 26D treatment as main factors. Degrees of freedom (df), sums of squares (SS), mean squares (MS) F values, P values are presented; Table S2. Impact of CPB damage and *Bacillus subtilis* 26D treatment on fresh (A) and dry (B) weights of shoots and roots of potato plants on the 9th day post damage caused by CPB. Data were analyzed using two-way ANOVA with CPB damage and B. subtilis 26D treatment as main factors. Degrees of freedom (df), sums of squares (SS), mean squares (MS) F values, P values are presented; Table S3. Impact of CPB damage and *Bacillus subtilis* 26D treatment on phytohormones level in potato plants. Data were analyzed using two-way ANOVA with CPB damage and B. subtilis 26D treatment as main factors. Degrees of freedom (df), sums of squares (SS), mean squares (MS) F values, P values are presented.

Author Contributions: A.S.: conceptualization, visualization, writing—original draft preparation, funding acquisition; S.V.: investigation; G.B.: investigation, validation; I.M.: writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by RSF № 20-76-00003 "Physiological basis of the formation of symbiotic relationships of potato plants with endophytic bacteria of the genus *Bacillus*".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available in a publicly accessible repository.

Conflicts of Interest: The authors declare no conflict of interest.

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Excessive Nitrogen Fertilization Favors the Colonization, Survival, and Development of *Sogatella furcifera* via Bottom-Up Effects

Zaiyuan Li †, Bo Xu †, Tianhua Du, Yuekun Ma, Xiaohai Tian, Fulian Wang * and Wenkai Wang *

Forewarning and Management of Agricultural and Forestry Pests, Hubei Engineering Technology Center, Institute of Entomological Science, College of Agriculture, Yangtze University, Jingzhou 434025, China; zaiyuanli01@163.com (Z.L.); 15327394823@163.com (B.X.); dutianhua01@163.com (T.D.); harris_m@163.com (Y.M.); xiaohait@sina.com (X.T.)

* Correspondence: wangfl_hb@163.com (F.W.); wwk@yangtzeu.edu.cn (W.W.)

† Zaiyuan Li and Bo Xu contributed equally to this work.

Abstract: Fertilization can trigger bottom-up effects on crop plant–insect pest interactions. The intensive use of nitrogen fertilizer has been a common practice in rice production, while the yield has long been challenged by the white-backed planthopper, *Sogatella furcifera* (Horváth). High nitrogen fertilization can facilitate *S. furcifera* infestation, however, how nitrogen fertilizer leads to high *S. furcifera* infestation and the nutritional interactions between rice and *S. furcifera* are poorly understood. Here, we evaluated the effects of various levels of nitrogen fertilizer application (0–350 kg/ha) on rice, and subsequently on *S. furcifera* performance. We found that higher nitrogen fertilizer application: (1) increases the preference of infestation behaviors (feeding and oviposition), (2) extends infestation time (adult lifespan), and (3) shortens generation reproduction time (nymph, pre-oviposition, and egg period), which explain the high *S. furcifera* infestation ratio on rice paddies under high nitrogen conditions. Moreover, high nitrogen fertilizer application increased all tested rice physical indexes (plant height, leaf area, and leaf width) and physiological indexes (chlorophyll content, water content, dry matter mass, and soluble protein content), except for leaf thickness, which was reduced. Correlation analysis indicated that the specific rice physical and/or physiological indexes were conducive to the increased infestation behavior preference, extended infestation time, and shortened generation reproduction time of *S. furcifera*. The results suggested that nitrogen fertilizer triggers bottom-up effects on rice and increases *S. furcifera* populations. The present study provides an insight into how excess nitrogen fertilization shapes rice–planthopper interactions and the consequent positive effect on *S. furcifera* infestation.

Keywords: rice-planthopper; nitrogen levels; bottom-up effects; nutritional interaction; IPM

1. Introduction

Plant–arthropod interactions are thought of as an important question in ecological research, which can provide insight into the dynamics of ecological communities and the mechanisms that shape interactions in complex food webs [1,2]. Plant–arthropod interactions can be markedly shaped by bottom-up forces, which are affected by abiotic factors and can in turn influence the performance of insect herbivores [1,3–7]. Plant nutrients could impact the performance of herbivorous insects via changes in plant quality in terms of nutritional and defensive aspects and determine bottom-up effects on plant–insect herbivore interactions. Among the plant nutritional factors that influence the performance of insect herbivores to a crop is total nitrogen [8–10].

Due to a large difference in nitrogen content between herbivorous insects and plant tissues, the nitrogen-containing nutrients of host plants are frequently considered as a limiting resource for the population development of herbivore insects [11]. The nitrogen

content of plants is often regulated by nitrogen fertilizer application [11]. Insufficient nitrogen input to plants was shown to impair the performance of herbivore insects, which was termed the "nitrogen limitation hypothesis" [5,12]. The harmful effects on the performance of herbivorous insects may be caused by the accumulation of high plant allelochemicals and toxic substances found on nitrogen-deficient plants [4,5,10]. Nitrogen-rich plants have positive effects on herbivore insects' performance, probably owing to deposition-induced improvements in host plant chemistry, which can significantly affect the structure and nutrition of plants [13–15]. Most current studies have shown support for the "nitrogen limitation hypothesis" [16–18]. However, several studies show that the development and survival of herbivore insects could respond negatively to nitrogen inputs to host plants, which undermined the generality of the "nitrogen limitation hypothesis" [19–22].

Rice (*Oryza sativa* L.) is one of the top five carbohydrate crops for the world's human population, especially in Asia. It is a major staple food, which supports more than three billion people and represents 50% to 80% of their daily calorie intake [23]. Part of the progress in rice production has resulted from a large amount of chemical fertilizer input, especially nitrogen (N) [24]. Low soil nitrogen level, especially in depleted croplands, is considered a major limitation for the growth of rice [16]. Therefore, the intensive use of nitrogen fertilizer has been a common approach for pursuing higher crop yields, and nitrogen application in rice production has shown a gradually increasing trend, as high as 350 kg/ha in many Asian regions, exceeding the reasonable amounts of 150 to 250 kg/ha [25,26]. However, rice is attacked by about 800 species of insect pests in the field and postharvest storage [27]. The white-backed planthopper, *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae), one of the most destructive migratory pests of rice, poses a substantial threat to rice production. Notably, Zhou et al. [28] found that the infestation ratio of *S. furcifera* was higher in paddies with high nitrogen fertilizer application. However, how high nitrogen fertilization level leads to high *S. furcifera* infestation and the nutritional interactions between rice and *S. furcifera* are poorly understood.

In the present study, we hypothesize that rice subjected to varying nitrogen inputs may trigger bottom-up effects on behavior as well as population fitness of *S. furcifera*. To test our hypothesis, we set up a trophic "rice-planthopper" system to carry out a series of bioassays under laboratory conditions, including (1) feeding and oviposition preferences of *S. furcifera*, (2) the life parameters of *S. furcifera*, (3) the physical and physiological indexes of rice, and (4) the relationships between behavior preferences, life parameters of *S. furcifera,* and physical and physiological indexes of rice with different nitrogen fertilizer applications. With these, we provide an insight into how excess nitrogen fertilization shapes rice–planthopper nutritional interactions and the consequent potential positive effect on *S. furcifera* infestation. Furthermore, these results may help to optimize the integrated pest management (IPM) of *S. furcifera* by nitrogen fertilizer manipulation.

2. Results

2.1. Feeding and Oviposition Preferences of S. furcifera Adults

The high amount of nitrogen fertilizer applied to rice plants had a significant effect on the feeding behavior of *S. furcifera* ($F = 11.546$, $df = 5, 29$, $p < 0.001$; Figure 1A). The adults of *S. furcifera* preferred to feed on N200, N250, and N350 rice plants, compared with N0 rice plants, and the adults' settling selection ratio increased by 32.33%, 20.22%, and 23.67% on rice from N200, N250, and N350, respectively.

The high nitrogen fertilizer levels also influenced the oviposition behavior of the adult females ($F = 7.302$, $df = 5, 17$, $p = 0.002$; Figure 1B). Among all the rice plants provided as options, the females preferred to lay eggs on the N250 and N350 rice plants, laying 677.78% and 791.36% respectively, more eggs than on N0 rice plants.

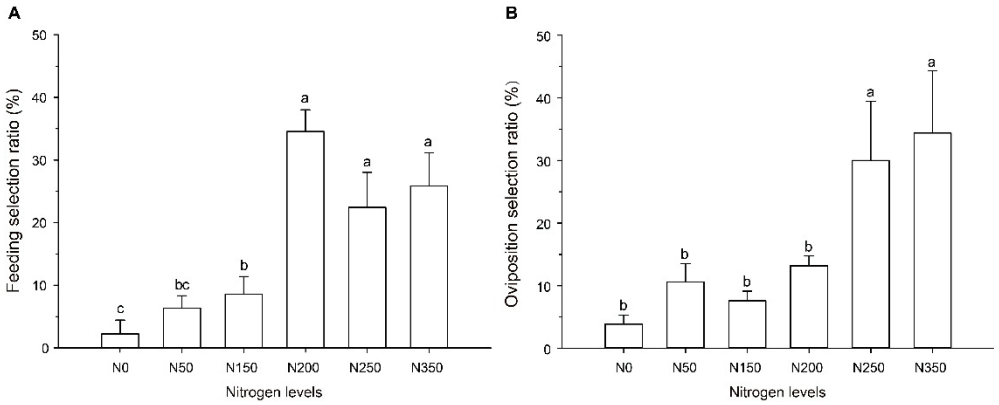

Figure 1. Feeding and oviposition preference of *S. furcifera* on rice of different nitrogen levels. (**A**) Feeding preference of *S. furcifera* on rice of different nitrogen levels. Feeding selection ratio of each nitrogen fertility level is expressed as a percent of the adults settling on a specific fertilizer level plant. (**B**) Oviposition preference of female on rice of different nitrogen levels. Oviposition selection ratio of each nitrogen fertility level is expressed as a percent of the eggs laid on a specific fertilizer level plant. In panels **A** and **B**, values (means ± SE) labeled with different letters are significantly different at $p < 0.05$ (one-way ANOVA, LSD multiple range test, $p < 0.05$). N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six nitrogen fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

2.2. Life Parameters of S. furcifera on Rice with Different Nitrogen Fertilizer Levels

2.2.1. Nymph Period

The nymph period differed in rice with different nitrogen fertilizer applications ($F = 24.837$, $df = 5, 245$, $p < 0.001$; Table 1) and the nymph period was shortened with nitrogen fertilizer application. The nymph period of *S. furcifera* raised on the N0 rice was the longest, being significantly longer ($p < 0.001$) than nymphs fed on the N50, N150, N200, N250, and N350 rice. The shorter nymph periods were observed on the nymphs raised on the high-nitrogen (N250 and N350) rice plants.

Table 1. Life parameters of *S. furcifera* feeding on rice with different nitrogen fertilizer applications.

Fertility Levels Rice	Nymph Period (days)	Adult Longevity (days)	Female Preoviposition Period (days)	Egg Period (days)	Total Pre-Oviposition Period (days)
N0	16.32 ± 0.31 a	9.19 ± 0.97 c	4.50 ± 0.50 a	7.46 ± 0.08 a	28.27 ± 0.5 a
N50	14.74 ± 0.27 b	11.46 ± 1.41 bc	4.00 ± 0.00 ab	7.40 ± 0.10 a	26.15 ± 0.2 b
N150	13.25 ± 0.19 d	14.96 ± 0.93 a	3.75 ± 0.25 abc	7.23 ± 0.05 b	24.23 ± 0.25 c
N200	13.98 ± 0.23 c	15.14 ± 0.84 a	2.75 ± 0.25 c	6.94 ± 0.05 c	23.67 ± 0.25 c
N250	13.30 ± 0.20 d	14.43 ± 1.07 a	3.25 ± 0.25 bc	6.72 ± 0.02 d	23.27 ± 0.25 c
N350	13.28 ± 0.24 d	13.26 ± 1.09 ab	3.75 ± 0.75 abc	6.81 ± 0.03 c	23.84 ± 0.75 c

Values are means ± standard error (SE). Different letters following the means in the same column denote significant difference at $p < 0.05$ via LSD multiple range tests. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively. Total pre-oviposition period is the duration from egg to first oviposition.

2.2.2. Longevity of Adults

There was a significant increase in the adult longevity of *S. furcifera* with high nitrogen fertilizer application ($F = 4.815$, $df = 5, 243$, $p < 0.001$; Table 1). Adult longevity of the insects raised on N0 rice was the shortest, which was significantly lower than the longevity of the adults raised on the N150 ($p < 0.001$), N200 ($p < 0.001$), N250 ($p < 0.001$), and N350 ($p = 0.011$) rice plants.

2.2.3. Mortality Dynamic of Adults

Adult mortality varied in *S. furcifera* insects raised on rice with different nitrogen fertilizer application levels (Figure 2). The relationship between the length of feeding

and adult cumulative mortality ratio was determined by a logistic relationship and was described by the nonlinear regression (Tables 2 and 3). The mortality ratio of the adult was highest in the insects reared on the N0 and N50 rice plants (Figure 2). When causing 50% adult cumulative mortality, the feeding day (7.9 days) was the shortest on the N0, whereas the feeding days were longer when adults were fed on the rice of N50, N150, N200, N250, and N350 (Table 4). Ingestion of high-nitrogen rice extended the lifespan of the adult stage. In addition, the time causing 50% adult cumulative mortality was delayed by 6.2, 6.2, 5.7, and 4.7 days when adults fed on rice of N150, N200, N250, and N350 compared with that on N0, respectively. The time causing 90% adult cumulative mortality was observed to be the shortest on N0 rice plants (Table 4).

2.2.4. Pre-Oviposition Period of Female

The pre-oviposition period lasted the longest in the females fed on the N0 rice plant, compared with those fed on the N200 ($p = 0.007$) and N250 ($p = 0.044$) (Table 1).

2.2.5. Egg Period

The developmental duration of the eggs of *S. furcifera* was established. The developmental period ($F = 45.891$, $df = 5, 882$, $p = 0.000$; Table 1) was significantly shortened by nitrogen fertilizer application. The longest developmental period was recorded for eggs from females fed N0 rice plants. Developmental durations of eggs were significantly shorter for eggs laid by females fed with the N150 ($p = 0.002$), N200 ($p < 0.001$), N250 ($p < 0.001$), and N350 ($p < 0.001$).

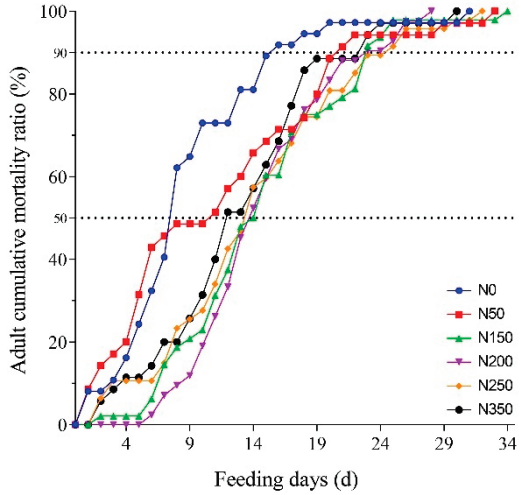

Figure 2. Adult cumulative mortality of *S. furcifera* on rice with different nitrogen fertilizer applications.

Table 2. Curve equation from a regression between adult cumulative mortality and feeding days on rice with different nitrogen fertilizer applications.

Fertility Levels Rice	Regression Equation	*R*	$R_{0.05}$	$R_{0.01}$
N0	Y = 96.719/(1 + Exp(2.834 − 0.369X))	0.994	0.355	0.456
N50	Y = 100.548/(1 + Exp(1.771 − 0.174X))	0.988	0.344	0.442
N150	Y = 99.076/(1 + Exp(3.737 − 0.266X))	0.996	0.339	0.436
N200	Y = 95.850/(1 + Exp(4.761 − 0.344X))	0.997	0.374	0.479
N250	Y = 99.038/(1 + Exp(3.148 − 0.233X))	0.998	0.349	0.449
N350	Y = 99.755/(1 + Exp(3.391 − 0.269X))	0.998	0.361	0.463

The regression equation was analyzed using SPSS. Y is the adult cumulative mortality of *S. furcifera*. X is the feeding days of *S. furcifera*. *R* is the coefficient of determination. $R_{0.05}$ is the coefficient of determination at 95%, $R_{0.01}$ is the coefficient of determination at 99%. *R* greater than $R_{0.05}$ and $R_{0.01}$ shows a strong relationship between adult cumulative mortality and feeding days. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

Table 3. Estimated parameters, confidence intervals, and R^2 for a logistic model of adult cumulative mortality fitted to experimental data of feeding *S. furcifera* on rice with different nitrogen fertilizer applications.

Parameter	Cumulative Mortality Ratio		
	Estimated	(95% CI)	R^2
N0			
A	96.719	(94.734, 98.705)	
b	2.834	(2.500, 3.168)	0.988
k	0.369	(0.326, 0.413)	
N50			
A	100.548	(96.161, 104.936)	
b	1.771	(1.546, 1.996)	0.977
k	0.174	(0.149, 0.200)	
N150			
A	99.076	(96.573, 101.580)	
b	3.737	(3.405, 4.069)	0.992
k	0.266	(0.241, 0.292)	
N200			
A	95.850	(93.189, 98.511)	
b	4.761	(4.319, 5.203)	0.994
k	0.344	(0.310, 0.379)	
N250			
A	99.038	(97.055, 101.021)	
b	3.148	(2.971, 3.324)	0.996
k	0.233	(0.218, 0.248)	
N350			
A	99.755	(97.642, 101.867)	
b	3.391	(3.168, 3.614)	0.996
k	0.269	(0.250, 0.289)	

A, b, and k represent the parameters in the logistic equation (Y = A/(1 + Exp (b − k X))). 95% CI, 95% confidence intervals. R^2, the coefficient of determination. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

Table 4. Time estimates for 50% and 90% of adult cumulative mortality.

Fertility Levels Rice	Time Estimates			
	50% Adult Cumulative Mortality		90% Adult Cumulative Mortality	
	Days	(95% CI)	Days	(95% CI)
N0	7.9	(5.1, 10.6)	14.7	(9.3, 20.1)
N50	10.1	(7.1, 13.2)	22.5	(16.5, 28.5)
N150	14.1	(12.0, 16.3)	22.7	(18.5, 26.9)
N200	14.1	(11.7, 16.5)	21.8	(17.1, 26.5)
N250	13.6	(12.1, 15.1)	23.4	(20.4, 26.3)
N350	12.6	(10.8, 14.5)	20.9	(17.2, 24.6)

CI, confidence interval. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

2.2.6. Biological Cycle Duration

The development duration from egg to first oviposition varied significantly with different nitrogen fertilizer applications ($F = 22.456$, $df = 5, 23$, $p < 0.001$; Table 1). The average total pre-oviposition period varied from 28.27 days on N0 to 23.27 days on N250. The total pre-oviposition period of *S. furcifera* fed with nitrogen application (N50–N350) rice plants was significantly shorter than N0 rice ($P_{\text{N50-vs-N0}} = 0.002$; $P_{\text{N150-vs-N0}} < 0.001$; $P_{\text{N200-vs-N0}} < 0.001$; $P_{\text{N250-vs-N0}} < 0.001$; $P_{\text{N350-vs-N0}} < 0.001$). However, the total pre-oviposition periods had no significant difference when *S. furcifera* was fed with N150, N200, N250, and N350 rice plants ($p > 0.114$).

2.3. Rice Physical Parameters with Different Nitrogen Fertilizer Applications

2.3.1. Plant Height

The effects of nitrogen application on plant height are listed in Table 5. The heights of the rice plant were significantly affected by the nitrogen application levels ($F = 8.577$,

df = 5, 59, $p < 0.001$). The lowest plant height was measured in the N0 rice, which was significantly lower than that of the rice plants of N150 ($p < 0.001$), N200 ($p < 0.001$), N250 ($p < 0.001$), and N350 ($p < 0.001$).

Table 5. Responses of rice physical parameters to nitrogen fertilizer application.

Fertility Levels Rice	Plant Height (cm)	Leaf Area (cm^2)	Leaf Width (cm)	Leaf Thickness (mg/cm^2)
N0	34.3 ± 2.0 b	2.0 ± 0.2 d	0.4 ± 0.0 d	12.2 ± 0.3 a
N50	39.1 ± 1.9 b	2.2 ± 0.1 cd	0.4 ± 0.0 cd	11.0 ± 0.7 b
N150	49.2 ± 2.7 a	2.8 ± 0.1 ab	0.6 ± 0.0 ab	10.1 ± 0.3 bc
N200	48.9 ± 2.2 a	2.4 ± 0.1 bc	0.5 ± 0.0 bc	10.6 ± 0.5 bc
N250	49.9 ± 1.9 a	2.9 ± 0.1 a	0.6 ± 0.0 a	9.7 ± 0.2 c
N350	46.8 ± 2.5 a	3.1 ± 0.1 a	0.6 ± 0.0 a	10.0 ± 0.2 bc

Values are means ± standard error (SE). Different letters following the means in the same column denote significant difference at $p < 0.05$ via LSD multiple range tests. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

2.3.2. Leaf Area, Width, and Thickness

The rice leaf area significantly varied with the levels of nitrogen fertilizer application ($F = 9.864$, *df* = 5, 23, $p < 0.001$; Table 5). The smallest leaf area measured was 2.0 cm^2 in the N0 rice plant, which had significantly smaller leaf areas than that of N150, N200, N250, and N350 plants ($p < 0.036$). The largest leaf areas measured were 2.9 and 3.1 cm^2 in the N250 and N350 rice plants, respectively.

Similarly, the differences in leaf width were evident when the rice was provided with different amounts of nitrogen fertilizer ($F = 9.864$, *df* = 5, 23, $p < 0.001$; Table 5). The leaf width of rice significantly increased with the increase of nitrogen fertilizer application.

Nitrogen fertilizer application greatly reduced the leaf thickness of rice ($F = 5.291$, *df* = 5, 23, $p = 0.004$; Table 5). The leaf thickness of the N0 rice was measured as 12.20 mg/cm^2, which was significantly thicker than the rice plants amended with nitrogen fertilizer ($p < 0.044$).

2.4. Rice Physiological Parameters with Different Nitrogen Fertilizer Applications

2.4.1. Leaf Chlorophyll Content (SPAD)

There was a significant increase in the leaf chlorophyll content (SPAD) with increased levels of nitrogen fertilizer amendment ($F = 37.482$, *df* = 5, 59, $p < 0.001$; Table 6). It was highest in the N250 and N350 treatments and significantly dropped in the N0 treatment ($p < 0.001$).

2.4.2. Water Content, Dry Matter Mass, and Soluble Protein Content

Water content in the main stems of the rice plant increased significantly ($F = 10.240$, *df* = 5, 29, $p < 0.001$; Table 6) with the increased amount of nitrogen fertilizer available to rice plants. The water content of the N0 rice was significantly lower than that of other rice plants given nitrogen fertilizer ($p < 0.037$). The water content in N200, N250, and N350 was significantly higher than N50 ($p < 0.026$).

Dry matter mass also differed significantly ($F = 21.501$, *df* = 5, 29, $p < 0.001$; Table 6) among the rice plants receiving various nitrogen fertilizer treatments, and nitrogen fertilizer application increased the rice ground dry matter, with the highest in the N200 and N350 and the lowest in N0 rice plants.

Rice leaf soluble protein contents significantly differed across the six nitrogen fertilizer treatments ($F = 11.849$, *df* = 5, 23, $p = 0.000$; Table 6). It was higher in the N350 and N250 rice leaves than in the others, and it steadily declined in the leaves of rice plants grown in the soil with no or less nitrogen fertilizer incorporated.

Table 6. Responses of rice physiological parameters to nitrogen fertilizer application.

Fertility Levels Rice	Chlorophyll Content (SPAD)	Water Content (%)	Dry Matter Mass (mg)	Soluble Protein Content (mg/g)
N0	25.8 ± 0.5 e	77.0 ± 0.4 c	311.9 ± 29.1 d	14.2 ± 0.5 d
N50	28.7 ± 0.7 d	78.9 ± 0.8 b	1077.5 ± 108.6 c	15.6 ± 0.1 c
N150	33.5 ± 0.7 c	80.3 ± 0.5 ab	1615.9 ± 84.5 ab	15.5 ± 0.4 c
N200	34.9 ± 0.8 bc	80.8 ± 0.4 a	1840.5 ± 87.9 a	16.3 ± 0.3 bc
N250	35.9 ± 0.9 ab	81.7 ± 0.4 a	1305.7 ± 214.0 bc	16.7 ± 0.4 ab
N350	37.5 ± 0.8 a	80.8 ± 0.7 a	1707.8 ± 122.8 a	17.7 ± 0.1 a

Values are means ± standard error (SE). SPAD represents rice plants' chlorophyll content. Different letters following the means in the same column denote significant difference at $p < 0.05$ via LSD multiple range tests. Percentage data were arcsine square-root-transformed, and homogeneity of variance of all data was tested before ANOVA. N0, N50, N150, N200, N250, and N350 represent the rice plants grown from the six fertility levels (0, 50, 150, 200, 250, and 350 kg/ha), respectively.

2.5. Relationships between Feeding and Oviposition Preferences, Life Parameters of S. furcifera, and Parameters of Rice with Different Nitrogen Fertilizer Applications

The relationships between feeding and oviposition preferences, life parameters of *S. furcifera,* and the plant parameters of rice with different nitrogen fertilizer levels were determined with the Pearson correlation analysis (Table 7). The results indicated a significant positive correlation between the feeding preference and chlorophyll content (SPAD). Oviposition preference had significant positive correlations with leaf area, leaf width, and soluble protein content. The nymph period had significant negative correlations with the plant height, leaf area, leaf width, leaf thickness, chlorophyll content (SPAD), water content, dry matter mass, and soluble protein content. Adult longevity had significant positive correlations with plant height, chlorophyll content (SPAD), water content, and dry matter mass; however, it had a negative correlation with leaf thickness. The female pre-oviposition period indicated only a significant negative correlation with plant height and water content. There were significant negative correlations between the egg period with chlorophyll content (SPAD), water content, and soluble protein content.

Table 7. Pearson's correlation coefficients and *p*-values between *S. furcifera* and rice responses to different nitrogen fertilizer applications.

S. furcifera Responses Parameters	Rice Responses Parameters							
	Physical Parameters				Physiological Parameters			
	Plant Height	Leaf Area	Leaf Width	Leaf Thickness	Chlorophyll Content (SPAD)	Water Content	Dry Matter Mass	Soluble Protein Content
Feeding selection ratio	0.716 (0.110)	0.512 (0.299)	0.512 (0.299)	−0.574 (0.234)	0.817 (0.047)	0.771 (0.073)	0.763 (0.078)	0.794 (0.059)
Oviposition selection ratio	0.539 (0.270)	0.823 (0.044)	0.823 (0.044)	−0.73 (0.100)	0.794 (0.059)	0.712 (0.113)	0.470 (0.347)	0.898 (0.015)
Nymph period	−0.940 (0.005)	−0.920 (0.009)	−0.920 (0.009)	−0.984 (0.000)	−0.923 (0.009)	−0.940 (0.005)	−0.881 (0.020)	−0.826 (0.043)
Adult longevity	0.979 (0.001)	0.687 (0.131)	0.687 (0.131)	−0.845 (0.034)	0.841 (0.036)	0.914 (0.011)	0.910 (0.012)	0.660 (0.154)
Female preoviposition period	−0.818 (0.047)	−0.413 (0.416)	−0.413 (0.416)	0.618 (0.191)	−0.728 (0.101)	−0.823 (0.044)	−0.784 (0.065)	−0.638 (0.173)
Egg period	−0.802 (0.055)	−0.804 (0.054)	−0.804 (0.054)	0.804 (0.054)	−0.924 (0.008)	−0.896 (0.016)	−0.668 (0.147)	−0.886 (0.019)

The correlations were then tested using Pearson's correlation coefficient, *p*-values shown in parentheses. The correlation level for significance is set to $p < 0.05$.

3. Discussion

In the present study, our results demonstrated that variation of nitrogen input to rice plants significantly affected the behaviors and life performance of *S. furcifera*. We found that: (1) higher nitrogen fertilizer application to rice plants increased the feeding and oviposition preferences of adults, and (2) nitrogen fertilizer application extended the adults' longevity and shortened the generation reproduction time of *S. furcifera*. These results indicated that altered nitrogen inputs to rice plants trigger a bottom-up effect on the biological traits of *S. furcifera* and then may explain the high infestation ratio of rice paddies with high nitrogen fertilizer by *S. furcifera*. The physical and physiological indexes change in host plants further explained the effects on the biological traits of *S. furcifera*. Nitrogen fertilizer application increased physical indexes (including plant height, leaf area, and leaf width) and physiological indexes (including chlorophyll content, water content, dry matter mass, and soluble protein content) in rice.

To understand insect behaviors, it is necessary to predict and prevent insect infestation outbreaks [29]. Feeding behavior is the first stage of acquiring nutrients for herbivorous insects, which is the foundation for a series of physiological activities to maintain the insect population. Oviposition behavior, the last stage of an herbivore's life cycle, has a crucial influence on the nutrition of the next-generation larvae [30–32]. Host orientation is a process by which organisms select host plants with potential nutritional resources, and it plays an important role in the process of feeding and oviposition [1]. In this study, nitrogen input to rice increased the feeding and oviposition preference of *S. furcifera* adults (Figure 1), especially in high nitrogen (N250, 350). In other words, excessive nitrogen fertilization favors the colonization of *S. furcifera*, which in turn leads to a high infection rate in the paddy. We assumed that the rice plant quality was improved under nitrogen input, thus causing the behavior preference. Indeed, we found that the physical and physiological parameters of rice were improved with nitrogen fertilizer applications (Tables 5 and 6). In particular, there was a significant correlation between the rice chlorophyll content (SPAD) and feeding preference after nitrogen application (Table 7). The chlorophyll content was positively correlated with nitrogen content and could be regarded as an indicator of the nitrogen content of plants [33]. Therefore, the nitrogen content of rice plants plays an important role in the feeding orientation of *S. furcifera* adults. Similarly, the leaf area, leaf width, and soluble protein content may play more important roles in oviposition orientation. In addition, most insects rely on olfactory cues to determine their host orientation [34]. We assumed that volatile organic compounds (VOCs) changed under nitrogen input, thus causing the behavior preference of *S. furcifera*. For example, tomato plants released less volatiles and increased the preference of *Bemisia tabaci* after high-nitrogen treatment [35].

For herbivorous insects, nitrogen plays a role in plant–herbivore interactions and the insects' development. Nitrogen fertilizer application on host plants not only influences many aspects of the behavior of herbivorous insects [36], but also impacts consumer survival, development rates, and fecundity by bottom-up effects after colonization [37,38]. The complete development of herbivorous insects depends on whether they obtain sufficient nutrients from the host plants [39,40]. In our study, nitrogen inputs positively affect *S. furcifera* survival and development (Table 1). In other words, excessive nitrogen fertilization favors the survival and development of *S. furcifera*. Furthermore, the rice nutrients (including chlorophyll content, water content, dry matter mass, and soluble protein content) were significantly increased in high nitrogen. Organic nitrogen (including amino acids and proteins) is considered as a limiting nutrient factor for herbivorous insects. When herbivorous insects feed on nitrogen-deficient plants, their metabolism can be reduced or impaired during the critical growth period [5,8]. Here, the developmental duration of *S. furcifera* egg and nymph had significant negative correlations with rice chlorophyll content (SPAD) and soluble protein content (Table 7). Therefore, we suggest that chlorophyll content (SPAD) and soluble protein content were key regulated factors influencing *S. furcifera*. Besides, it is worth noting that under nitrogen fertilizer, the water

content has a significant correlation with all life parameters of *S. furcifera*. Similarly, Han et al. [5] reported that *Tuta absoluta* had a lower survival rate and longer development time when fed on the host plants subjected to drought. We assumed that *S. furcifera* had difficulty in obtaining enough water for optimal development, thus causing a slow development when fed on the nitrogen-deficient rice plants. In addition, we found that the development time of *S. furcifera* from egg to first oviposition did not significantly shorten when fed on N150–N350 rice plants. Similar results were found in *Aphis gossypii* [41] and *Trialeurodes vaporariorum* [42]. The total pre-oviposition period of *T. vaporariorum* was not significantly different fed on a nitrogen concentration of more than 310 ppm in the nutrient solution of the tomato plants [42]. We assumed that the *S. furcifera* may excrete excess nutrients from its body when they fed on N150–N350 rice plants [43].

Mechanical barriers can reduce herbivores' ingestion performance, as they increase plant resistance [44]. Zheng et al. [45] reported that *Laodelphax striatellus* preferred to feed on rice with a large leaf area. In this study, we found that the plant height, leaf area, and leaf blade width of rice significantly increased after nitrogen fertilizer application, which may help the *S. furcifera* to select its optimal host. Besides, leaf thickness is related to a decline in the ingestion ability of sap-sucking insects [46–48]. For example, *Aphis gossypii* needed to spend more time penetrating thick cotton leaves, which resulted in reduction of feeding efficiency [49]. Similarly, the penetration of mature lemon leaves by *Parabemisia myricae* nymphs appeared to be inhibited by cuticle thickness [50]. In this study, we found that there was a negative correlation between the leaf thickness and the levels of nitrogen fertilizer application in rice. We suggest that the leaf thickness of rice decreases after nitrogen fertilizer application, and it reduces the mechanical barrier on ingestion by *S. furcifera*. Therefore, *S. furcifera* can get more nutrition from high-nitrogen plants, which subsequently favors the survival and development of *S. furcifera*.

Adult life expectancies significantly increased when raised on rice with nitrogen fertilizer application, showing a positive correlation between adult lifespan and nitrogen fertilizer application. The times causing 50% and 90% adult cumulative mortality were extended for *S. furcifera* in the rice with high nitrogen fertilizer application (Table 4). Huang and Feng [51] found that *S. furcifera* adults consume more than nymphs, indicating that the adult stage was the most harmful period for rice. Our results suggest that nitrogen input may extend the adult lifespan and thus cause a longer rice infestation time, which may be one of the reasons why the rice with high-nitrogen fertilizer application experienced more damage than the one with low application [52]. In addition, the rice with high-nitrogen fertilizer application shortened the length of the nymph, pre-oviposition, and egg stages, thus shortening the life cycle of *S. furcifera*, however, increasing fecundity and total populations. Therefore, this may be another reason for the high infestation rate of *S. furcifera* populations in the paddy with high-nitrogen fertilizer.

To conclude, we suggest that nitrogen input to rice improves plant quality (physiological and physical indexes) and further facilitates the colonization, survival, and development of *S. furcifera*. Our findings support the "plant vigor hypothesis", that vigorous plants are the best and preferred host plants for herbivore insects [5,53].

Insufficient nitrogen input to plants triggered negative bottom-up effects on *S. furcifera*, which was consistent with the "nitrogen limitation hypothesis" [54,55]. Manipulating nitrogen fertilization inputs could help to optimize agronomic practices against herbivore pests [7]. This method could gain optimal ecological and economic benefits based on a little damage to plants and negligible crop yield losses. Based on a complex agronomic database covering a wide range of climate conditions, soil types, and field managements, Che et al. [56] found that in most rice-growing areas, rice grain yield with nitrogen inputs of 200–250 kg/ha was higher than other nitrogen rate inputs. Here, the preference of infestation behaviors (feeding and oviposition), the infestation time (adult lifespan), and generation reproduction time (nymph, pre-oviposition, and egg period) of *S. furcifera* in 200 kg/ha nitrogen input were sub-optimal compared to high-nitrogen inputs (250 and 350 kg/ha). Therefore, we propose 200 kg/ha nitrogen input as an optimal fertil-

izer level in rice field managements since this will create a win-win situation. Moreover, rice cultivars that are tolerant to nitrogen-deficiency stress were deliberately selected in breeding, underpinning agronomic leverage for IPM packages against *S. furcifera* by the bottom-up effect.

4. Materials and Methods

4.1. Preparation of Soil with Various Fertility Levels

Soil for planting the rice plants was obtained from the fields at the Yangtze University Experimental Station for Crop Pests (30°35′50″ N, 112°14′77″ E) in Hubei, China. To prepare the planting soil with specific fertility levels, the dry soil was mixed with urea fertilizer (soluble nitrogen $\geq$ 46.4%, Hubei Sanning Chemical Co., Ltd., Zhijiang, China) at the rate of 0, 50, 150, 200, 250, and 350 kg/ha. This converted to 0, 0.033, 0.098, 0.131, 0.164, or 0.229 g urea respectively, mixed with 456.50 cm^3 dry soil to fill each pot (diameter 7 cm, height 14 cm). Thirty pots for each of the above six fertility levels were prepared for planting rice, and a batch of 180 pots was prepared every 10 days to ensure enough pots were available for planting rice plants for the experiment.

4.2. Rice Plants' Preparation for Nitrogen Treatments

Germinated rice seeds of the variety Taichung Native 1 (TN1), susceptible to *S. furcifera*, were individually planted (one seed per pot) in each prepared pot already filled with soil amended with fertilizer. Thirty single-seed pots were prepared and repeated every ten days, for each fertility level, to ensure enough rice plants for the experiment, and they were then kept in cages, measuring 60 $\times$ 80 $\times$ 60 cm^3, under laboratory conditions (26 $\pm$ 1 °C, 70–80% RH, and 14 h light). Seeded pots were watered as needed and the water level was kept below the upper edge of each pot. Pesticides were not used on the rice plants throughout the experiment. The rice plants grown from the six fertility levels were called N0, N50, N150, N200, N250, and N350 rice, respectively. The rice plants were used in the experiments, 35 days after transplanting (DAT).

4.3. Insects

A colony of *S. furcifera* was kindly provided by Dr. Hou of the Chinese Academy of Agricultural Sciences. To maintain and increase the test insect population, they were reared on the N0 rice plants without exposure to any insecticide. The rice plants were enclosed in 80-mesh screen cages (30 $\times$ 30 $\times$ 60 cm^3) placed in a climate-controlled rearing room with 26 $\pm$ 1 °C, 70 $\pm$ 5% RH, and a photoperiod of 14L:10D.

Under the same conditions mentioned above, *S. furcifera* was also reared on N50, N150, N200, N250, and N350 rice plants in order to obtain test insects that were reared at various nitrogen fertility levels. Newly hatched nymphs (<24 h) and newly emerged adults (<24 h) were used in the experiments, except where otherwise specifically noted.

4.4. Feeding and Oviposition Preferences

To prepare for the experiment testing rice nitrogen levels on the feeding and oviposition preference of *S. furcifera*, one 35 DAT potted rice plant from each of the six nitrogen levels (N0, N50, N150 N200, N250, and N350) was randomly selected to be included in the test. The selected rice plants, 6 in total, were trimmed such that only the main stem remained in each pot. Then, the cleaned 6 potted rice plants were placed within a cage (30 $\times$ 30 $\times$ 60 cm^3, made of iron wire frame and covered with 80-mesh nylon netting) and randomly arranged in a circular shape.

Thirty newly emerged macropterous adults (<24 h, 15 females and 15 males) from the *S. furcifera* colony reared on N0 rice plants were released from a 9 cm Petri dish placed into the center of the cage. After 96 h from release, the number of adults on each of the 6 plants was recorded, and the feeding preferences of each nitrogen fertility level were expressed as a percent of the adults settling on a specific plant. The feeding preference was calculated as: Feeding preference = The number of adults settling on a specific fertilizer

level plant/Total number of tested adults × 100. This experiment was repeated 5 times and for each repetition, the location of the plants in the cage was rotated to avoid the effect of other factors.

The same arrangement was used to observe the adult's oviposition preferences. Fifteen pairs of newly emerged macropterous adults from the *S. furcifera* colony reared on N0 rice plants were released for the oviposition preferences test. The experiment was replicated 5 times. After that, the number of first instars emerging and unhatched egg on a specific plant to count the total number of eggs laid. The leaf midribs and leaf sheaths of each nitrogen level rice were dissected under a binocular microscope to count the unhatched egg. The tests were performed in a climate chamber (RZH-260A, Hangzhou Huier Instrument Equipment Co., Ltd., Hangzhou, China) at 26 ± 1 °C, 70 ± 5% relative humidity (RH), and a photoperiod of 14L:10D. The oviposition preference was calculated as: Oviposition preference = The number of eggs laid on a specific fertilizer level plant/Total number of eggs laid × 100.

4.5. Life Parameters

A 4 cm-long rice leaf section cut from the top third or fourth leaf, starting 5 cm away from the leaf tip of a 35 DAT potted rice plant receiving a fertility treatment, was placed in a glass tube (1.0 cm in diameter, 7.5 cm in height) filled with 1 mL water. Then, one first instar nymph (<24 h) reared from the N0 rice plants was transferred into the glass tube and the opening was sealed with a sponge to prevent escape. The leaf section in the glass tube was replaced daily with a freshly cut leaf section from the same fertility treatment. Using the same procedure, rice leaves from all 6 fertility levels were prepared to evaluate the host's impact on the life parameters of *S. furcifera*. All glass tubes containing rice leaf sections and individual nymphs were kept in a climate chamber at 26 ± 1 °C, 70 ± 5% RH, and a photoperiod of 14:10 (L:D) h. The nymphs' developmental stage and mortality were recorded daily until they died or became adults, and the emerged adults were then sexed. The observation of nymphal survival was repeated at least 50 times for each of the 6 fertility levels.

The effects of nitrogen fertility on the longevity of newly emerged adults (<24 h) reared from the N0 rice plants were evaluated using the same arena and method as those for the nymphs, except that the newly emerged adults were transferred into the glass tubes at the beginning of the experiment. The longevity observation was replicated at least 35 times for each of the 6 fertility levels, and the ratio of males to females tended to be 1:1.

Newly emerged adult males and females (<24 h) reared from the rice plants of a fertility level were paired, and they were transferred into a glass tube (5 cm in diameter, 40 cm in height) housing a single 35 DAT rice plant of fertility treatment. The top of the glass tube was sealed with a sponge. The rice plant was replaced daily with a new plant from the same fertility treatment. Similar setups were prepared for all 6 fertility treatments. These experimental setups were used to observe the pre-oviposition and egg development periods. The observation of pre-oviposition was replicated four times for each treatment, with one pair of adults as a replicate. The egg developmental period observation was replicated at least 48 times, for each fertility treatment, with 1 egg as a replicate. The tests were performed in a climate chamber at 26 ± 1 °C, 70 ± 5% relative humidity (RH), and a photoperiod of 12L:12D.

4.6. Rice Physical Parameters

The above-ground heights of 35 DAT rice plants were measured, using a ruler, for 10 rice plants from each fertility level. To obtain the blade thickness and leaf areas, the following procedures were performed. From the main stem of a 35 DAT potted rice plant, the third leaf from the top was chosen. A 5 cm-long rice leaf section from the leaf tip was cut and discarded. Another 5 cm-long rice leaf section was cut from the same leaf and then weighed and photographed individually. The images were analyzed using the

Image-Pro Plus v6.0 software to obtain the areas of the leaf blade sections. The leaf blade thickness was expressed as the weight per unit area (mg/cm^2) according to Luo et al. [46]. The leaf blade width was calculated as Blade width = Blade area/Blade length. Four 5 cm leaf blade sections from the rice plants of each fertility treatment were used to obtain the blade area, thickness, and width data. All tests were replicated 10 times for each fertility treatment.

4.7. Rice Physiological Parameters

The chlorophyll content was positively correlated with nitrogen content and can be regarded as an indicator of the nitrogen content of plants [33]. The rice leaf tissue chlorophyll content was assessed using an electronic portable chlorophyll meter (SPAD-502, Minolta camera Co., Osaka, Japan) and expressed as the SPAD value. The mean of three SPAD readings measured by the chlorophyll meter was obtained from each of the top third leaves of ten 35 DAT rice plants of various fertility levels from 9:00 to 10:00 a.m. The tests were performed in a glasshouse, under natural light and temperature conditions, and repeated 10 times for each fertility treatment.

To obtain water content, the main stems from ten 35 DAT rice plants for each fertility level were cut from the base flush with the soil and weighed individually. These stems were immediately placed in an oven at 110 °C for 30 min and then at 80 °C for 48 h (when constant weight was achieved). The tests were repeated 5 times in each fertility treatment. The water content was calculated as: Water content = (Fresh weight − Dry weight)/Fresh weight × 100. The dry weight was regarded as dry matter mass.

The concentration of the soluble protein of 35 DAT rice plant leaves of various fertility levels was determined using the Coomassie Blue method [57], with bovine serum albumin as a standard, and the absorbance of the samples was measured at 595 nm using the UV-VIS Spectrophotometer. This procedure was repeated 5 times in each fertility treatment.

4.8. Statistical Analysis

The adult mortality ratio (Adult mortality ratio = Larval mortality/Total number of tested larvae × 100) in all the rice of the six nitrogen levels (N0, N50, N150, N200, N250, and N350) tested was transformed to probability units and analyzed using the logistic regression (Y = A/(1 + Exp (b − k X))), where X is the feeding days in each rice of different nitrogen levels and Y is the adult mortality ratio in probability units. The feeding days and adult mortality ratio data were fitted using probability analysis to estimate the number of feeding days that induced death in 50% and 90% of adults, respectively.

Percentage data (including feeding and oviposition preference, water content) were arcsine square-root-transformed, and all data fit normal distributions. Then, the homogeneity of variance of all data was tested before one-way analysis of variance (ANOVA). All data were subjected to ANOVA, followed by the least significant difference (LSD) multiple range test ($p < 0.05$) for determining the significant differences between the treatments. We investigated the relationships between *S. furcifera* and rice responses to nitrogen fertilizer application, and the relationships were then tested using the Pearson correlation coefficient. All the statistical analyses were performed using SPSS 17.0.

5. Conclusions

Nitrogen fertilizer can trigger bottom-up effects on rice and *S. furcifera*, favoring the colonization, survival, and development of this insect. Nitrogen inputs to rice increase the preference of infestation behaviors (feeding and oviposition), extend infestation time (adult lifespan), and shorten generation reproduction time (nymph, pre-oviposition, and egg period), causing a high infestation rate of *S. furcifera* populations in the paddies with high-nitrogen fertilizer. Various nitrogen inputs to rice plants affected the biological traits of the *S. furcifera* and these effects were due to the changes of rice physical and/or physiological indexes. Our results shed insight into how excess nitrogen fertilization shapes

rice–planthopper nutritional interactions. Negative bottom-up effects in insufficient nitrogen input may help to optimize the IPM program of *S. furcifera* by nitrogen fertilizer manipulation. Further work will investigate on how nitrogen fertilization inputs shape rice–planthopper–natural enemies (i.e., *Cyrtorhinus lividipennis* and *Anagrus nilaparvatae*) nutritional interactions and further optimize agricultural practices by bottom-up effects.

Author Contributions: Conceptualization, W.W., F.W. and Z.L.; software, Z.L. and Y.M.; investigation, Z.L., B.X. and T.D.; resources, X.T.; writing—original draft preparation, Z.L. and B.X.; writing—review and editing, W.W. and F.W.; visualization, Z.L. and B.X.; funding acquisition, W.W. and F.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Open Fund of Hubei Collaborative Innovation Centre for Grain Industry, grant number 2015MS023.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Tools to Tie: Flower Characteristics, VOC Emission Profile, and Glandular Trichomes of Two Mexican *Salvia* Species to Attract Bees

Claudia Giuliani [1,2], Manuela Giovanetti [3,4,*], Daniela Lupi [5], Marco Palamara Mesiano [5], Renata Barilli [2], Roberta Ascrizzi [6], Guido Flamini [6] and Gelsomina Fico [1,2]

1 Department of Pharmaceutical Sciences, University of Milan, Via Mangiagalli 25, I-20133 Milan, Italy; claudia.giuliani@unimi.it (C.G.); gelsomina.fico@unimi.it (G.F.)
2 Ghirardi Botanical Garden, Department of Pharmaceutical Sciences, University of Milan, Via Religione 25, I-25088 Toscolano Maderno, Brescia, Italy; renatabarilli@yahoo.it
3 Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal
4 CREA—Research Centre for Agriculture and Environment, Via di Saliceto 80, 40128 Bologna, Italy
5 Department of Food, Environmental and Nutritional Sciences, University of Milan, Via Celoria 2, I-20133 Milan, Italy; daniela.lupi@unimi.it (D.L.); palamaramesiano.marco@gmail.com (M.P.M.)
6 Department of Pharmacy, University of Pisa, Via Bonanno 6, I-56126 Pisa, Italy; roberta.ascrizzi@gmail.com (R.A.); guido.flamini@farm.unipi.it (G.F.)
* Correspondence: manuela.giovanetti@crea.gov.it

Received: 2 November 2020; Accepted: 20 November 2020; Published: 25 November 2020

Abstract: A plant can combine physical and chemical tools to interact with other organisms. Some are designed for pollinator attraction (i.e., colors and volatile organic compounds-VOCs); others can act to discourage herbivores (i.e., non-glandular trichomes). Few studies fully address available tools in a single species; notwithstanding, this information can be pivotal in understanding new interactions out of the home range. We characterized flower traits, emission profiles of constitutive compounds from flowers and leaves, micro-morphology of the glandular trichomes, and listed flower visitors of two Mexican bird-pollinated *Salvia* species (*S. blepharophylla* and *S. greggii*), growing in an Italian botanical garden. Flowers were highly variable in their morphometric characteristics. In both species, four trichome morphotypes with similar histochemistry and distribution were documented for leaves and flowers except the calyx abaxial side. The vegetative emission profiles were qualitatively more complex than the floral ones; however, common compounds occurring in high relative percentages were β-caryophyllene and germacrene D. Floral bouquets were dominated by limonene and β-pinene in *S. greggii* and by 1,8-cineole in *S. blepharophylla*. Two potential (non-bird) pollinators were especially abundant: small bees belonging to the genus *Lasioglossum* and large bees belonging to the species *Xylocopa violacea*. Our study highlights the plasticity of these plants, as well as tools that can be conveniently used to establish novel interactions.

Keywords: bees; glandular trichomes; *Salvia blepharophylla*; *Salvia greggii*; Lamiaceae; VOCs; pollinators

1. Introduction

In the course of evolution, plants have developed different strategies to attract or repel other living organisms. As attractants, the synthesis of colored substances and the production of volatile organic compounds (VOCs) by glandular trichomes are among the most investigated ones [1–6]. As deterrents, epidermal structures may act as physical barriers [7], while the emission of volatiles can be a first "warning shout" against predators [3]. A deeper knowledge of how a plant can employ

these tools will greatly help in understanding the evolutionary perspective of ecosystem working conditions. However, information is still scattered and often incomplete, even for single well-studied species. Our study arises in this framework. We were interested in sketching the potential of a given plant species in terms of tools applied to attractive/deterrent performances and their plasticity in actual plant–animal interactions. Therefore, we selected two exotic species with an evolutionary path possibly in contrast with the local occurring interactions. The species, growing in a botanical garden in Italy (well-acclimatized since they were planted many years in advance) belong to the genus *Salvia* (Lamiaceae): *Salvia blepharophylla* Brandegee ex Epling (Figure S1) and *Salvia greggii* A.Gray (Figure S2). They are both native to Mexico, with *S. greggii* extending its home range to the southern region of Texas. They are procumbent ornamental plants widely used in horticulture, with distinctive attractive red flowers for bird pollination [8].

S. blepharophylla (eyelash-leaved sage) can reach 60 cm in height at full bloom. The leaves have a serrate margin with evident long trichomes along the edges. Flowers are arranged in loose whorls and are red in color with an orange undertone. *S. greggii* (autumn sage) may reach 1.20 m in height, but it is very variable in size and flower color due to the existence of numerous cultivars. In the wild, leaves are typically ovate and glabrous, with an entire margin, and flowers, gathered in racemes, are scarlet red. The leaf glandular *indumentum* has been investigated in *S. blepharophylla* [9], whereas an ultrastructural characterization of the terpene-producing trichomes exists for *S. greggii* [10]. On the contrary, literature data on the characterization of spontaneously emitted VOCs are lacking for both target species. In the native range, both species are referred to as bird pollinated and possess flowers with an active working lever mechanism [8]. However, a single record of the hummingbird *Calipte costae*, observed on white varieties of *S. greggii* cultivated in California [8], has been reported. In the botanical garden, an entomological survey pointed out that these species also attract bees, at lower abundance and variety [11] in comparison to bee-pollinated *Salvia* species. Overall, the genus *Salvia* is well studied for what concerns the pollination strategies of the numerous species: literature data on the pollination ecology in relation to floral morphology are rich ([12] and literature therein), even though there are no specific studies or inferences correlating such interactions with secondary metabolite production.

The current study provides a comprehensive view of the potential of these species (phytochemical characterization of the VOCs spontaneously emitted from leaves and flowers; presence and distribution of glandular trichomes) and how they may be linked to interactions occurring out of the home range in contrast with previously established pollination syndromes [13,14].

2. Results

2.1. Floral Traits and Pollinator Monitoring

The flowers of the target species are arranged in flowering shoots (Figure 1a). Attraction is enhanced by the dense growth form of the shrubs and by the many simultaneously flowering shoots. The target species present common traits: (i) a hooded upper lip, in which the fertile thecae are hidden; (ii) stigmas protruding out of the upper lips; (iii) a distinct thinner basal part of the corolla tube and a rapidly expanding distal part; (iv) the two lever-like modified stamens typical of the genus; (v) the upper connective arm, bearing a fertile theca, located within the upper lip, while the lower one is long; (vi) the nectary located at the base of the ovary with the nectar accumulating in the thin basal part of the corolla tube.

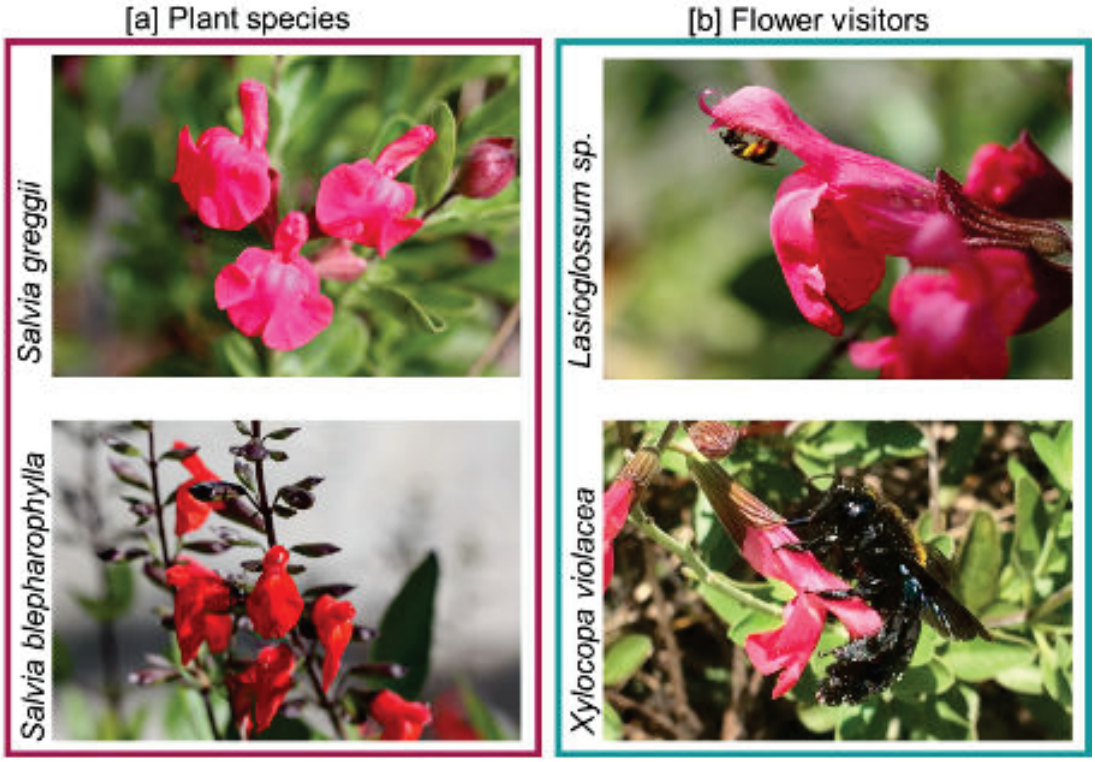

Figure 1. Photographic records of the flowers of [a] *S. blepharophylla* ([a] top) and *S. greggii* ([a] bottom) and of the flower visitors [b] *Lasioglossum* spp. ([b] top) and *Xylocopa violacea* ([b] bottom).

The two species differ in the following floral characters: (i) the color of the corollas looks red, but their tone is different: it is cold with an orange undertone in *S. blepharophylla*, and warmer with a touch of magenta in *S. greggii*; (ii) the floral proportions differ in the overall flower size, shape, and orientation of the lower lip (slightly reflexed in *S. blepharophylla*, deflexed in *S. greggii*) and among the six examined floral morphological traits (Table 1).

Table 1. Floral morphometric variability of the six examined parameters in *Salvia blepharophylla* and *Salvia greggii*: (a) calyx length; (b) flower length; (c) upper lip length; (d) lower lip length; (e) length of the corolla tube (measured as the distance between the top of the ovary—where the nectary is typically located—and where the petals separate); (f) relative length of the upper lip to the corolla tube. The numbers (millimeters) are mean values, with standard error in parentheses.

	a	**b**	**c**	**d**	**E**	**f**
S. blepharophylla	17.79 [1] *(0.13)*	27.21 [1] *(0.42)*	10.60 [1] *(0.42)*	18.63 [1] *(0.38)*	16.61 [1] *(0.84)*	0.64 [1] *(0.05)*
S. greggii	10.50 [2] *(0.26)*	24.66 [2] *(0.77)*	10.44 [1] *(0.39)*	16.40 [2] *(0.35)*	14.22 [2] *(0.38)*	0.73 [2] *(0.01)*

[1,2] Different superscript numbers indicate significant differences (Tukey's HSD, $p \leq 0.05$) for the same parameter (within the same column).

Plant visual display of *S. greggii* and *S. blepharophylla* changed across the blooming period. The total size of *S. greggii* plants ranged from 63 to 120 cm^2, on average occupying 82.67 ± 16.72 cm^2 with 32.00 ± 21.10 inflorescences. The average size of *S. blepharophylla* plants was 104.56 ± 11.54 cm^2 with 43.78 ± 34.39 inflorescences. There were 1–3 available flowers during anthesis on each inflorescence of *S. greggii* and 2–5 flowers on *S. blepharophylla*. Even though, at the inflorescence level, *S. blepharophylla* displayed more flowers than *S. greggii* during most of the observations, the overall display (estimated

number of flowers during anthesis on the whole plant during the season) was not significantly different (*t* test, unequal variances assumed = −1.336, df = 9.78, $p = 0.2119$).

Various pollinators, belonging to Hymenoptera, were present in the study area (reported in [11]). However, both sages mainly attracted small *Lasioglossum* spp. and large *Xylocopa violacea* (Figure 1b). Based on the frequency and constancy of their visits, we can certainly conclude that these visits were not random or generic events. The bees repeatedly visited the flowers and collected the resources. We counted 47 visits on *S. greggii* and 134 on *S. blepharophylla*, distributed across 10 different days and 97 patch records. A clear difference between the two species emerged with more visits paid to *S. greggii* than to *S. blepharophylla.* The presence of bees on flowers changed during the season (Figure 2); the trend was not related to each *Salvia* species, but to the time of the year as shown by the overall increase during summer months.

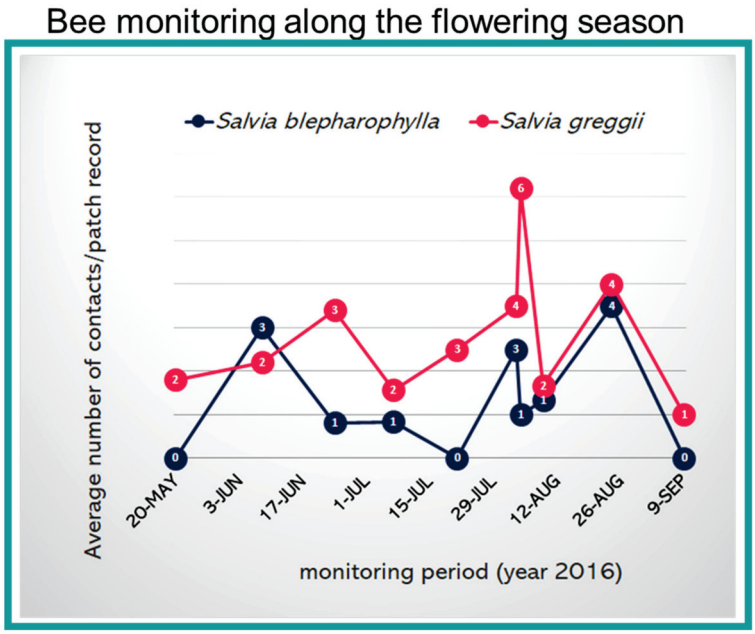

Figure 2. Bee monitoring during the flowering season on the two *Salvia* species. Bee species were summed, with daily average number of contacts during a patch record reported in the graph. In total, we recorded 47 visits on *S. greggii* and 134 on *S. blepharophylla*, distributed across 10 different days and 97 patch records.

Moreover, the two *Salvia* species were visited by different bees: *S. blepharophylla* only attracted *Lasioglossum* spp. (100% of records), whereas *S. greggii* attracted *Lasioglossum* spp. (84.3% records) plus *X. violacea* (15.7% records). There was a statistical difference in the number of flowers visited for each species: more *S. greggii* flowers were visited (*t* test, unequal variances assumed = 3.390; df = 38.32; $p = 0.0016$). *Lasioglossum* spp. visited both species, mainly interested in the collection of pollen. The same individual may have visited between 1 and 10 flowers during a single foraging bout on *S. greggii*, and between 1 to 3 flowers on *S. blepharophylla*. *X. violacea* visited only *S. greggii* and was interested solely in nectar collection (100% of visits, $n = 16$ records). During our observations, the bee did not contact the pollen, since it accesses it from behind the corolla. However, we cannot state that the bee never visits the plant in a legitimate way, since it may occur at a lower frequency than the opposite behavior. Finally, some individuals were observed visiting the same flower repeatedly.

2.2. Glandular Indumenta and Volatile Organic Compounds (VOCs)

The glandular *indumenta* of the target species exhibit both peltate and capitate trichomes (Figure 3a–m, Table 2). Four main morphotypes were recognized:

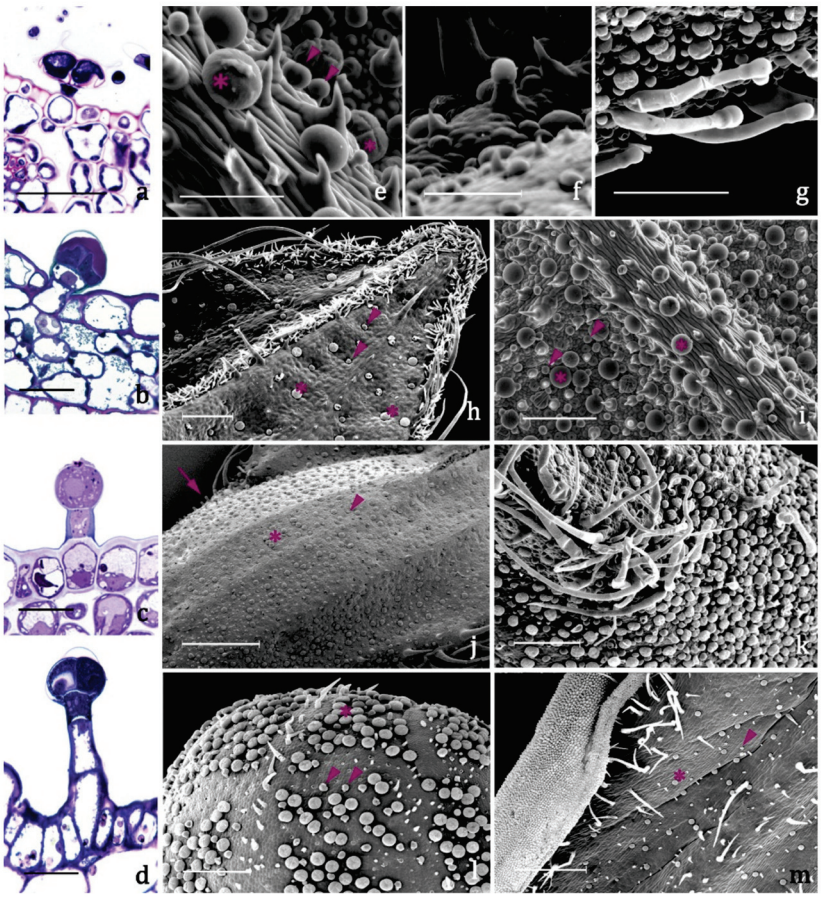

Figure 3. Trichome morphotypes and distribution pattern in *S. blepharophylla* and *S. greggii*. a-d. Light Microscope micrographs. Transverse sections of: (a) type A, peltate trichome. (b) type B, small capitate trichome. (c) type C, medium capitate trichome. (d) type D, long capitate trichome. e-m. Scanning Electron Microscope micrographs. (e). Types A peltate and B short capitate trichomes. (f) Type C medium capitate trichome. (g) Type D long capitate trichome. (h) Leaf abaxial surface of *S. greggii*. (i) Leaf abaxial surface of *S. blepharophylla*. (j) Calyx abaxial surface of *S. blepharophylla*. (k) Calyx abaxial surface of *S. greggii*. (l) Corolla abaxial side of *S. greggii*. (m) Corolla abaxial surface of *S. blepharophylla*. ***Scale bars*** = 25 μm (a–d), 100 μm (e, f, i), 200 μm (g, h, k, l), 500 μm (j, m). **Symbols:** asterisk = type A peltate trichome; arrowhead = type B short capitate trichome; arrow: type C medium capitate trichome.

Table 2. Distribution patterns of the morphotypes of the glandular *indumen*a on leaves and flowers.

	Trichome Morphotype	Leaf		Calyx		Corolla	
		Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
S. blepharophylla	A	+	+	+	-	+	+
	B	+	+	+	+	+	+
	C	-	-	+	-	-	-
S. greggii	A	+	+	+	+	+	+
	B	+	+	+	+	+	+
	D	-	-	+	-	-	-

- type A (Figure 3a,e, Table 2), present on leaves and inflorescences of both species (Figure 3h–m), is a typical peltate trichome, constituted by a basal epidermal cell, a neck cell, and by a 4–cellular glandular head surrounded by a large subcuticular space in which the secretion is stored. The responses to all the lipophilic stains were positive as well as to Ruthenium Red and AlCl3,

indicating the presence of terpenes and of major polysaccharide and flavonoid derivatives (Table 3).

- type B (Figure 3b,e, Table 2) is a short capitate trichome, widespread on both the vegetative and the reproductive organs of both examined species (Figure 3h–m). It is constituted by a basal epidermal cell, a neck-stalk cell, and by a glandular head of 2–4 cells surrounded by a wide subcuticular space. Generally, these trichomes present an exclusive polysaccharide secretion released through the intact cuticle (Table 3).
- type C (Figure 3c,f, Table 2) is a medium capitate trichome present only on the calyx of *S. blepharophylla* (Figure 3j). It is made up of one epidermal cell, one stalk cell, one neck cell and a globose head of 1–2 secretory cells surrounded by a storage chamber. The secretion tested positive to all the lipophilic stains, particularly the NADI reagent, indicating that they are exclusive terpene producers (Table 3).
- type D (Figure 3d,g, Table 2) is a long capitate trichome occurring only on the calyx of *S. greggii* (Figure 3k). It is composed by 1–2 epidermal cells, two stalk cells, one neck cell and by a head of 2–4 secretory cells. The secreted material stored in the subcuticular space tested positive only to the lipophilic dyes, indicating the exclusive production of terpenes (Table 3).

Besides glandular trichomes, abundant protective uniseriate trichomes were observed in both species, especially at the edge of the leaves, on the foliar lamina, and along the veins of calyces. In *S. blepharophylla,* they also occurred on the abaxial side of the corolla upper lip. These projections generally point apically toward the top of the organ and are oriented at acute angles to the epidermal surface.

Table 3. Results of the histochemical tests performed on the glandular trichomes. Symbols: (-) absent; (±) scarce, (+) intense, and (++) very intense.

Staining Procedure	Target Compounds	Observed Colour	*S. blepharophylla*			*S. greggii*		
			Type A	Type B	Type C	Type A	Type B	Type D
Nile Red	Neutral lipids	Golden-yellow	++	-	++	++	-	++
Fluoral yellow-088	Total lipids	Yellow to orange	++	-	++	++	-	++
NADI reagent	Terpenes	Violet-blue	++	-	++	++	-	++
$FeCl_3$	Polyphenols	Emerald-green	+	-	-	++	-	-
$AlCl_3$	Flavonoids	Blue-green	+	-	-	+	-	-
Ruthenium red	Acid polysaccharides	Pinkish to red	+	+	-	+	+	-
Alcian blue	Muco- polysaccharides	Pale-blue	+	+	-	+	+	-

As for VOCs, the flower headspace of *S. blepharophylla* was rich in oxygenated monoterpenes (66.40%) and in sesquiterpene hydrocarbons (22.61%) (Table 4). Among monoterpenes, the most abundant compound was 1,8-cineole (7, 45.68%, Table 4), followed by isobornyl formate (32, 8.56%). The two main sesquiterpene hydrocarbons were β-caryophyllene (50, 6.84%) and germacrene D (64, 5.01%). Among the exclusive compounds accounting for more than 1.00%, we detected isobornyl acetate (37, 1.91%) and trans-cadina-1(6),4-diene (61, 2.65%) (Table 4). In the leaf samples of *S. blepharophylla*, sesquiterpene hydrocarbons accounted for 54.95%, followed by oxygenated monoterpenes (18.83%) and oxygenated sesquiterpenes (13.80%). The most abundant compounds were β-caryophyllene (50, 11.07%), β-bourbonene (43, 10.43%), trans-α-bergamotene (53, 6.89%), and (Z)-β-farnesene (56, 6.82%). Methyl carvacrol (33, 10.68%) was a relevant oxygenated monoterpene, followed by linalool (14, 5.07%). Among the oxygenated sesquiterpenes, the principal one was (Z)-sesquilavandulol (81, 9.63%). Thirty-two exclusive compounds characterized the leaf profile: those occurring in higher relative amounts were linalool (14, 5.07%), trans-α-bergamotene (53, 6.89%), (Z)-β-farnesene (56, 6.82%), and (Z)-sesquilavandulol (81, 9.63%).

Table 4. HS-SPME profiles of leaves and flowers of *S. blepharophylla* and *S. greggii*.

	l.r.i.[a]	Compounds	*Salvia blepharophylla*		*Salvia greggii*	
			Relative Abundance (%)		Relative Abundance (%)	
			Flowers	Leaves	Flowers	Leaves
1	941	α-pinene	2.14	-[b]	2.27	3.23
2	954	camphene	-	-	-	0.57
3	982	β-pinene	2.44	-	14.32	24.96
4	993	myrcene	-	-	1.90	-
5	1005	α-phellandrene	-	-	0.45	-
6	1032	limonene	-	1.66	55.20	-
7	1034	1,8-cineole	45.68	-	-	19.56
8	1052	(*E*)-β-ocimene	-	3.71	-	-
9	1062	γ-terpinene	0.36	0.86	0.22	-
10	1070	*cis*-sabinene hydrate	-	-	0.25	0.34
11	1076	*trans*-linalool oxide (furanoid)	-	-	-	0.89
12	1088	terpinolene	-	0.44	0.42	-
13	1090	*cis*-linalool oxide (furanoid)	-	-	-	0.67
14	1101	linalool	-	5.07	-	2.11
15	1102	nonanal	0.56	-	-	-
16	1104	α-thujone	-	0.41	-	-
17	1134	*cis*-limonene oxide	-	-	0.20	-
18	1140	nopinone	0.75	0.67	-	-
19	1141	*trans*-limonene oxide	-	-	3.63	0.08
20	1143	camphor	2.95	-	-	2.09
21	1156	*iso*borneol	-	-	-	0.17
22	1158	sabinaketone	-	0.47	-	-
23	1162	*trans*-pinocamphone	-	-	0.15	0.26
24	1167	borneol	-	-	-	0.26
25	1170	δ-terpineol	-	-	-	0.05
26	1178	4-terpineol	1.23	-	0.19	-
27	1187	(*Z*)-3-hexenyl-butyrate	0.97	-	-	-
28	1192	methyl salicylate	-	-	-	0.14
29	1195	γ-terpineol	-	-	0.20	-
30	1202	*trans*-dihydro carvone	-	-	0.19	-
31	1204	decanal	0.63	1.88	-	0.23
32	1232	isobornyl formate	8.56	0.73	-	-
33	1241	methyl carvacrol	5.32	10.68	0.61	0.23
34	1259	linalool acetate	-	0.80	-	-
35	1272	*n*-decanol	-	0.08	-	-
36	1283	(*E*)-anethole	-	0.92	-	-
37	1285	isobornyl acetate	1.91	-	-	-
38	1300	*n*-tridecane	-	0.56	-	-
39	1340	δ-elemene	-	0.65	-	-
40	1351	α-cubebene	-	-	-	0.05
41	1368	cyclosativene	-	-	0.20	0.24
42	1376	α-copaene	0.86	-	0.53	2.6
43	1384	β-bourbonene	1.20	10.43	0.83	2.74
44	1390	β-cubebene	0.28	-	0.23	0.4
45	1391	7-*epi*-sesquithujene	-	0.77	-	-
46	1392	β-elemene	-	1.05	0.15	0.55
47	1400	*n*-tetradecane	-	0.23	-	-
48	1403	longifolene	0.41	-	-	0.16
49	1409	α-cedrene	0.68	0.28	-	-
50	1420	β-caryophyllene	6.84	11.07	5.73	5.59
51	1429	β-copaene	0.48	1.33	0.39	0.65
52	1432	β-gurjunene	-	-	0.41	6.74
53	1438	*trans*-α-bergamotene	-	6.89	-	-
54	1439	α-guaiene	-	-	-	0.12
55	1441	aromadendrene	-	-	0.35	0.15
56	1445	(*Z*)-β-farnesene	-	6.82	-	-
57	1455	(*E*)-geranyl acetone	0.41	0.41	-	-
58	1456	α-humulene	0.76	2.34	-	0.45
59	1461	*allo*aromadendrene	-	1.40	0.16	0.93
60	1462	*cis*-muurola-4(14),5-diene	-	0.20	0.27	0.21
61	1470	*trans*-cadina-1(6),4-diene	2.65	-	-	-
62	1477	γ-muurolene	0.79	-	1.48	10.2
63	1480	γ-curcumene	-	0.06	-	-
64	1481	germacrene D	5.01	4.22	6.37	7.22
65	1490	(*E,Z*)-α-farnesene	-	0.80	-	-
66	1491	*trans*-muurola-4(14),5-diene	-	-	0.16	-

Table 4. *Cont.*

	l.r.i.[a]	Compounds	*Salvia blepharophylla*		*Salvia greggii*	
			Relative Abundance (%)		Relative Abundance (%)	
			Flowers	Leaves	Flowers	Leaves
67	1492	valencene	0.46	-	-	0.51
68	1495	bicyclogermacrene	-	2.38	-	-
69	1496	γ-amorphene	-	-	-	0.13
70	1498	α-muurolene	-	-	0.30	1.08
71	1500	*n*-pentadecane	-	0.32	-	-
72	1502	γ-patchoulene	0.52	0.10	0.29	-
73	1507	(*E*,*E*)-α-farnesene	-	2.42	0.29	0.82
74	1513	*trans*-γ-cadinene	0.98	-	0.70	0.43
75	1524	β-sesquiphellandrene	-	1.73	-	-
76	1524	δ-cadinene	0.69	-	0.24	0.40
77	1549	elemol	-	0.25	-	-
78	1565	(*E*)-nerolidol	-	1.29	-	-
79	1575	germacrene D-4-ol	-	-	-	0.16
80	1576	spathulenol	-	2.16	-	-
81	1593	(*Z*)-sesquilavandulol	-	9.63	-	-
82	1595	guaiol	-	-	-	0.76
83	1600	*n*-hexadecane	-	1.28	-	-
84	1606	humulene epoxide II	-	0.14	-	-
85	1640	*epi*-α-cadinol	-	-	0.18	-
86	1693	juniperol acetate	-	0.33	-	-
87	1700	*n*-heptadecane	-	0.08	-	-
		Monoterpene hydrocarbons	4.94	6.67	74.78	28.76
		Oxygenated monoterpenes	66.40	18.83	5.42	26.71
		Sesquiterpene hydrocarbons	22.61	54.95	19.08	42.37
		Oxygenated sesquiterpenes	-	13.80	0.18	0.92
		Phenylpropanoids	-	0.92	-	-
		Apocarotenoids	0.41	0.41	-	-
		Other non-terpene derivatives	2.16	4.43	-	0.37
		Total identified (%)	**96.52**	**100.00**	**99.46**	**99.13**

[a] Linear retention indices on a DB5 column; [b] Not detected.

In *S. greggii*, the flower volatile profile was mainly rich in terpene hydrocarbons: monoterpenes accounted for 74.78% and sesquiterpenes for 19.08% (Table 4). The main component was limonene (6, 55.20%), followed by β-pinene (3, 14.32%); the two most abundant sesquiterpene hydrocarbons were germacrene D (64, 6.37%) and β-caryophyllene (50, 5.73%). Among the characterizing compounds exceeding 1.00%, myrcene (4, 1.90%) should be mentioned. The leaf samples of *S. greggii* were rich in terpene hydrocarbons: sesquiterpenes reached 42.37%, while monoterpenes accounted for 28.76%. The principal sesquiterpene hydrocarbons were γ-muurolene (62, 10.20%), germacrene D (64, 7.22%), β-gurjunene (52, 6.74%), and β-caryophyllene (50, 5.59%). Among monoterpenes, the most represented compounds were β-pinene (3, 24.96%) and 1,8-cineole (7, 19.56%). Twenty compounds were exclusively present in the leaf profile: the dominant ones were 1,8-cineole (7, 19.56%) and camphor (20, 2.09%) (Table 4).

Although monoterpenes dominated the floral emission profiles of both species, the overall composition appeared diverse. The same was true for the leaf headspace of the two species, where sesquiterpene hydrocarbons prevailed. Among the most abundant compounds emitted by the flowers, only β-pinene (3), methyl carvacrol (33), β-bourbonene (43), β-caryophyllene (50), and germacrene D (64) occurred in both profiles, even if in different relative abundances. In the case of the leaf headspace, α-pinene (1), linalool (14), decanal (31), β-elemene (46) β-caryophyllene (50), germacrene D (64), and (E,E)-α-farnesene (73) were the common compounds. Finally, methyl carvacrol (33), β-bourbonene (43) β-caryophyllene (50), β-copaene (51), and germacrene D (64) were present in the flower and leaf emission profiles of both species and were therefore ubiquitous.

3. Discussion

The flowers of *S. greggii* and *S. blepharophylla* are both typically ornithophilous [8], meaning they are characterized by a very long corolla, longer and larger than those of entomophilous species in the same genus, and red in color. The red color should not act as an attractive cue to bees, which are not

able to see this color. However, we cannot neglect the possible presence of UV-mediated attraction or the presence of reflectance peaks at wavelengths able to stimulate green or blue bee receptors [8].

According to the evolution towards bird pollination, some morphological modifications evolved to increase the distance between the nectar and the pollen: e.g., elongation of the corolla tube, exposure of the stigma, and reflexion of the lower lip [14]. All these traits co-occur in the two examined species, so that bees are supposedly excluded from resource collection: the elongation of the corolla tube impedes the relatively short proboscis of bees to reach the nectar, and also the pollen, concealed in the upper lip. When compared to existing groups [14] based on flower morphology, the two species belong to group I, the Lanceolata-type, which includes sages with a working lever mechanism. Our morphometric investigation revealed a large variability in terms of different tones of red, overall size, and reciprocal distances between the various floral whorls, even among flowers of the same plant. These differences could also account for the illegitimate visits of *X. violacea*. The absence of differences in the overall floral display indicates other features are involved in the attraction of different pollinators. Differences between the two species emerged regarding the average number of flowers visited during anthesis at any given moment and the tube length, much longer in *S. blepharophylla* than in *S. greggii*. These indicate the flexibility of structures, i.e., flowers may eventually be visited by otherwise unpredicted visitors. In fact, we recorded bees visiting these flowers, even if adopting alternative strategies. *Xylocopa violacea* collected only nectar, its visits being not legitimate [15]: therefore, current data advocate for this species not being a pollinator. However, legitimate visits are possible according to flower traits, and it may still be considered a potential pollinator. Some individuals have been observed repeatedly visiting the same flower, but data are needed to define if this occurs due to leftover nectar or mismatching of attractiveness and actual resource presence (signal-reward correlation) [16]. Harder [17] underlined that bees have restrictions on the tube length they can visit, based on the length of their own glossa. Considering its own length, *X. violacea* was potentially able to legitimately collect nectar from *S. greggii*. However, *X. violacea*, with a proboscis of about 13.00 mm long [18], was equally observed robbing other flowers with corollas within the range of compatibility of its ligula. It is acknowledged that robbing is a constant foraging strategy in this bee species [18] and it is a better explanation for the observed behavior than some incompatibility of bee–flower structures. *Lasioglossum* spp. were rarely recorded looking for nectar, legitimately trying to push the lever mechanism but with evident problems in tripping it due to the small size. However, individuals were very active in collecting pollen: their small size allowed them to hang directly from the anthers. These bees, transferring pollen from one flower to the other, can be considered potential good pollinators of these ornithophilous species.

The glandular *indumentum* is constituted by peltate and capitate trichomes. The peltate trichomes were widespread on all the epidermal surfaces and exhibited a broad four-celled head, as in other *Salvia* species [19]. The peculiar feature of type A trichomes is the exclusive production of polyphenols and flavonoids. These depositions could presumably contribute to the flower visual attractiveness towards some insects, as flavonoids absorb light in the UV range. We recorded two types of capitate trichome on each of the *Salvia* species: type B small capitate occurring on the whole plant epidermis of both species; type C medium capitate and type D long capitate exclusive of the calyx, of *S. blepharophylla* and *S. greggii*, respectively. Type B and C trichomes correspond morphologically to types I and II as described for *S. blepharophylla* leaves [9]. Type B small capitates resulted in exclusive pectic–polysaccharide producers. Inferring that these secretion droplets pass through the intact cuticle, Antunes and Sevinate-Pinto [20] hypothesized that these substances might act as a lubricant to facilitate plant-organ expansion. In type C and D trichomes, the secretory products were exclusively stained by the terpene indicator NADI reagent. The peculiar distribution of types C and D only on calyces is certainly remarkable: they presumably constitute the main sites for the production of the floral volatiles and appear to be mainly located on the abaxial surface, co-occurring with trichomes of types A and B. Therefore, while both leaf and corolla surfaces are comparable in relation to trichome distribution and emission patterns as well, calyces exhibited a peculiar condition: a qualitatively more complex productivity on the abaxial surface due to the presence of three different types of trichomes; an exclusive synthesis of polysaccharides on the adaxial side for

the occurrence of only type B. The complex production on the abaxial surface may be primarily related to the potential defensive role of the calyx (as the outermost whorl) in flower buds. Secondarily, at full blooming, the defensive role probably declines while the calyx and its resources enhance attraction.

With regards to the phytochemical data, the flower emission profiles are characterized by the occurrence of one or few exclusive compounds (Figure 4), which may differently affect the interactions between the flowers and the insect pollinators. In *S. greggii,* limonene is massively emitted, followed by β-pinene. In *S. blepharophylla* 1,8-cineole is very abundant, exceeding 45.0%, followed by isobornyl formate. Leaf emission profiles were instead qualitatively more complex. *S. greggii* had 11 compounds with a relative abundance exceeding 2.0%, compared to six in the flower emission profiles. In *S. blepharophylla,* 12 volatiles occurred with relative percentages greater than 2.0% in the leaves, with respect to eight in the flowers. The total profiles of the two species were qualitatively very diverse, excluding the presence of germacrene D, β-caryophyllene and methyl carvacrol that were common to all the analyzed samples. The role of volatiles in bee–plant interactions has been poorly studied due to difficulties in carrying out controlled experiments, and few bee species have been analyzed in relation to plant scents. As evidence of this, the existing information about the chemical cues involved in the attraction of *X. violacea* and *Lasioglossum* spp. to flowers is very rare and refers solely to the latter [21,22].

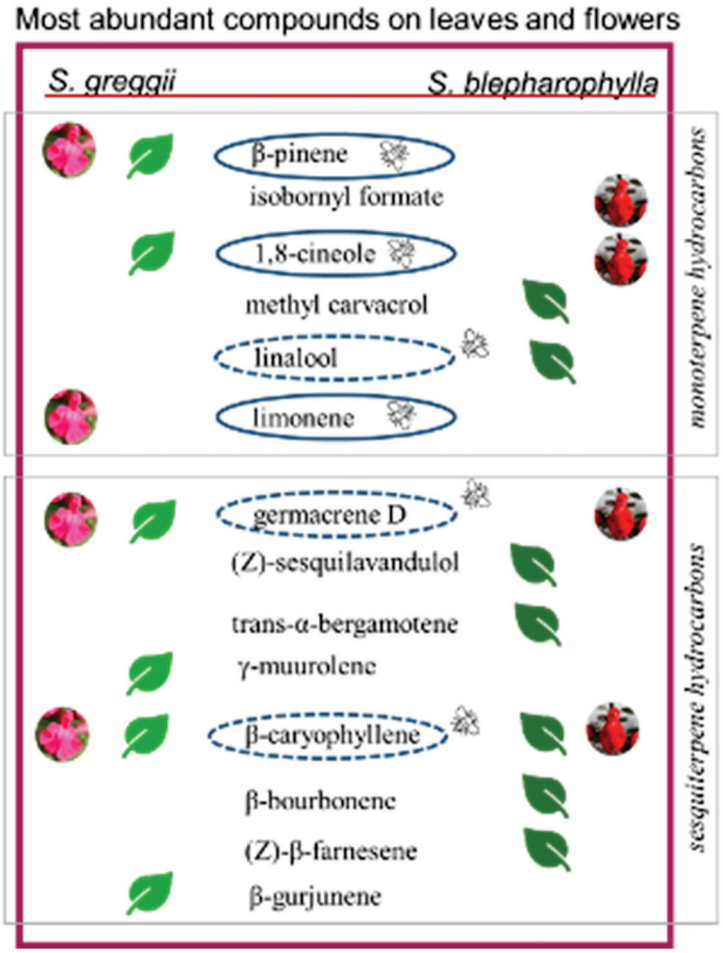

Figure 4. The most abundant compounds found in leaves and/or flowers of the *Salvia* species. Those compounds known from literature to elicit responses by insects are highlighted by continuous (attractive cue) or dotted (deterrent cue) circles.

The detection of two bee species in association with *S. greggii* and one with *S. blepharophylla* can be attributed to numerous factors. However, we can also infer that the presence, in their volatile profiles, of compounds emitted by other plants generally visited by these bees might have facilitated the first

contact among these native bees and the two *Salvia* species. Certainly, it can be stated that *X. violacea* and *Lasioglossum* spp. found these novel sources interesting, based on the frequency and constancy of their visits. It has been inferred that emissions rich in benzenoids or in linalool (and its oxides) could be an adaptation to a butterfly or to a generalist pollinator [22,23]. Flower attractiveness can be due to a single substance, even if it is more often associated with the total bouquet [24,25]. When the floral bouquet is dominated by a sole volatile in relatively large percentages, the pollination is often bee-mediated [26] (Table 5).

Table 5. Floral scent compounds eliciting positive behavioural responses in bees.

Compound	Bee Species	Reference
1,8-Cineole	Euglossini, *Bombus terrestris; Bombus vorticosus*	[26–29]
α-Pinene	Euglossini, *Apis mellifera;* Honeybees	[26,30]
β-Pinene	*Bombus*; Honeybees	[31–33]
Limonene	*Bombus*, Honeybees; *B. terrestris; B. vorticosus*	[29,31]
β-Caryophyllene	*Apis mellifera*;	[29,34]
α-Farnesene		[24]
(*E*,*E*)-α-Farnesene	*B. terrestris; B. vorticosus; Apis mellifera*	[29,31,35]
Linalool	Colletidae bees; Apidae; *Lasioglossum* spp.	[36]
(*E*)-β-Ocimene	Colletidae bees; Apidae; *Lasioglossum* spp.	[36]

S. greggii and *S. blepharophylla* emitted 1 –2 main compounds in their floral bouquets. Limonene and β-pinene, which are the main volatiles in *S. greggii* flowers, have been demonstrated to be involved in the flower attraction by bumblebees [32] (Table 5) and of Apidae Meliponinae [37]. In addition, 1,8-cineole, which characterizes the flowers of *S. blepharophylla*, appears to have a very important role in the attraction of different bees [26–28] (Table 5). According to Granero et al. [28], this compound is also an alarm pheromone for *Bombus terrestris*, which might explain the absence of this pollinator among *S. blepharophylla* visitors. Notwithstanding the intense attraction possibly played by the floral volatiles, we have to keep in mind the mechanical difficulties encountered by bees in handling these flowers, which may have lowered total visitation rates. The physical barriers may also involve the non-glandular and glandular indumenta. The impact of trichome density, length, and orientation on insect behavior and performance has been well documented for herbivores [38,39], while information on the influence on pollinators is scarce or lacking [40].

Finally, volatile biosynthesis is also a defensive response: the production of β-caryophyllene, germacrene D, and linalool can be induced by herbivory [41,42]. Linalool was exclusively emitted by the leaf samples of the studied species, while the other two compounds were also detected in the flower samples. The defensive role of reproductive organs is normally less important in comparison to that of leaves, even if it cannot be completely neglected: a synergistic flower–leaf action may be ascribed to the common emission of deterrent volatiles such as β-caryophyllene or germacrene D, thus ensuring widespread protection throughout the plant.

In conclusion, our study is the first combining macro- and micromorphological investigations with VOC analyses and direct records of flower visitors. Even if no experimental procedure could be implemented, the study gives a simultaneous prospect of co-occurring circumstances, which allows us to infer newly-established connections between exotic plant species and native bees. Our data highlighted the plastic learning capacity of the local bees, able to bypass physical barriers by adopting peculiar strategies to collect a resource. However, a certain degree of plasticity was certainly also displayed by the plants: notwithstanding their evolutionary path towards bird pollination, they retained some characteristics able to attract insect visitors. The next step would be evaluating the reciprocal benefit, in terms of resource collected for the bees and successful pollination events for the plants and place this in the context of pollination syndromes.

Exhaustively detailing the causes of the different attraction methods of *X. violacea* and the similar attraction by *Lasioglossum* spp. was beyond the scope of this paper. Speculating the substances that may elicit attraction is very difficult in the absence of direct and controlled experiments. Bees are extremely sensitive: for males, slight variability in the relative percentage of the different volatiles in the bouquets of pheromones can strongly impact the attraction potential. The same complexity can be expected by females attracted to flowers, and the complexity may also translate in the same compounds eliciting opposite responses. This was confirmed by the fact that the *S. greggii* bouquet was dominated by limonene and β-pinene: according to literature, these compounds are very attractive to honeybees and bumblebees (Table 5; Figure 4). However, during our surveys, none of these species was detected visiting the plant. Conversely, no information is available on the attraction elicited by 1,8-cineole, which is the dominating substance in *S. blepharophylla*. More multidisciplinary studies are needed in the future to indicate the possible importance of the given compounds and to establish corresponding experiments.

4. Materials and Methods

4.1. Plant Material, Floral Traits, and Pollinator Monitoring

4.1.1. Plant Material

Salvia blepharophylla and *Salvia greggii* are cultivated at the Ghirardi Botanical Garden (Toscolano Maderno, BS, Italy) of the Department of Pharmaceutical Sciences of the University of Milan. Plants were identified using the original protologues [43,44], and voucher specimens were deposited in the Herbarium of the Ghirardi Botanical Garden, University of Milan, Italy, under the accession codes UNIMI 0028/15 and 0029/15, respectively. Sampling of leaves and flowers was carried out simultaneously for the micro-morphological and phytochemical studies in June 2016. The macro-morphological investigation and pollinator monitoring were performed throughout summer 2016.

4.1.2. Flower Traits

Thirty randomly selected fully-opened flowers per species were dissected and measured using a digital caliper and a stereomicroscope. Six floral morphological traits per species were selected and measured [44]: (i) calyx length; (ii) flower length; (iii) upper lip length; (iv) lower lip length; (v) length of the corolla tube (measured as the distance between the top of the ovary—where the nectary is typically located—and where the petals separate); (vi) relative length of the upper lip to the corolla tube. The 30 replicates for each parameter were transformed using arcsine square root (arcsin $\sqrt{x}$) for normalization and then subjected to analysis of variance (ANOVA) to obtain mean values and confidence intervals ($\alpha = 0.05$). Averages were separated by Tukey's b post hoc test; $p < 0.05$ was used for the significance of differences between means. The statistical analyses were carried out using the JMP software package (SAS Institute, Cary, NC, USA).

4.1.3. Pollinator Monitoring

We listed flower visitors by randomly recording presence on the flowers on sunny days between 8:00 and 14:00 (local solar hour), through patch records (one observer in a fixed position in front of a *Salvia* plant) lasting 10 min and repeated 2–10 times during the day [45]. Data refer to 10 days, from May to September 2016 fortnightly: 48 patch records on *S. greggii* (totally 480 min) and 49 on *S. blepharophylla* (totally 490 min). Data were normalized according to the number of slots of each day. Plant size (expressed in cm as the projection of the canopy) and number of flowers, as well as weather conditions (descriptive: sunny or cloudy conditions) were recorded at the beginning of each day and of patch observed. Bees were visually identified at least at the genus level. Specimens were also collected for further determination at the species level. Each visit to a single flower was described as (i) resource

collected and (ii) number of approached flowers. The entire list of the pollinator fauna in the botanical garden is reported elsewhere [11].

4.2. Micro-Morphology of the Glandular Indumentum and Phytochemical Investigation (VOCs)

Leaves, floral buds, and fully open flowers were analyzed by means of scanning electron microscopy and light microscopy to observe: (i) the structure and distribution of the glandular *indumentum* on both vegetative and reproductive organs and (ii) the histochemical nature of the secreted substances. Ten replicates, similar in size, position, and developmental stage, were selected from different individuals for each plant part, in order to verify the consistency of the trichome morphotypes, distribution pattern, and histochemistry.

4.2.1. Scanning Electron Microscopy (SEM) and Light Microscopy (LM)

We combined SEM and LM analyses to describe the trichomes. For SEM samples, fresh leaf, bract, calyx, and corolla samples (5 mm2) were mounted on brass stubs. These samples were viewed in an ambient mode analysis with a QUANTA-200 FEI ESEM.

For LM samples, histochemical tests were performed on hand-cut fresh material to detect the presence of terpenes, lipids, muco-polysaccharides, and phenolics [46–50]. For all the histochemical methods, standard control procedures were carried out simultaneously. All the sections for histochemistry were examined under a light microscope Leitz DM-RB Fluo equipped with a digital camera Nikon DS-L1.

4.2.2. Headspace-Solid Phase Microextraction (HS-SPME) Analyses, Gas Chromatography–Mass Spectrometry (GC-MS) Analyses, and Peak Identification

Three leaves and five complete flowers were cut for each species and inserted into glass vials of suitable volume for the sampling. For HS-SPME sample analysis, Supelco SPME (Solid Phase Micro-Extraction) devices coated with polydimethylsiloxane (PDMS, 100 μm) were used to sample the headspace. SPME sampling was performed using the same new fibre, preconditioned according to the manufacturer instructions, for all the analyses. Sampling was accomplished in an air-conditioned room (22 ± 1°C) to guarantee a stable temperature. After the equilibration time, the fibre was exposed to the headspace for 30 min. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC–MS system. All the SPME sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each first SPME extraction and randomly repeated during each series. Quantitative comparisons of relative peaks areas were performed between the same chemicals in the different samples. Analyses of leaves were performed in triplicate. Due to their scarce availability, replicates were not considered for flowers.

Gas Chromatography with Electron Impact Mass Spectrometry (GC–EIMS) analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Injector and transfer line temperatures were kept at 250 °C and 240 °C, respectively; the oven temperature was programmed from 60 °C to 240 °C at 3 °C min^{-1}; the carrier gas was helium at 1 mL min^{-1}; splitless injection. The mass spectra were compared with those listed in the commercial libraries NIST 14 and ADAMS and in a home-made mass-spectral library, built up from MS literature [51,52] combined with data experimentally obtained from pure substances and commercial essential oils of known composition.

Supplementary Materials: The following botanical dissection drawings are available online at http://www.mdpi.com/2223-7747/9/12/1645/s1. They were drawn in graphite continuous tone to match the delicacy of the watercolour. We represented the androecium and gynoecium in white inside the corolla, to be shown clearly at first. Watercolour paper Moulin du Roy Canson, graphite Derwent, and watercolour Winsor & Newton were used. Figure S1: Botanical illustration of *Salvia blepharophylla* Brandegee ex Epling; Figure S2: botanical illustration of *Salvia greggii* A.Gray.

Author Contributions: Conceptualization, G.F. (Gelsomina Fico), C.G., M.G. and D.L.; methodology, C.G., M.G., D.L., R.A., G.F. (Guido Flamini) and G.F. (Gelsomina Fico); investigation, C.G., M.G., D.L., M.P.M., R.A., G.F. (Guido Flamini) and G.F. (Gelsomina Fico); resources, Giuliani, M.G., D.L., R.A., R.B., G.F. (Guido Flamini) and G.F. (Gelsomina Fico); data curation, C.G., M.G., D.L., R.A., G.F. (Guido Flamini) and G.F. (Gelsomina Fico); writing—original draft preparation, C.G. and M.G.; writing—review and editing, C.G., M.G., D.L., R.A., G.F. (Guido Flamini), G.F. (Gelsomina Fico); visualization, C.G., M.G., D.L. and G.F. (Gelsomina Fico); supervision and project administration, G.F. (Gelsomina Fico); funding acquisition, D.L. and G.F. (Gelsomina Fico). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by *Regione Lombardia*, under the Call for the "Enhancement of Museums" (lr. 25/2016, year 2019).

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Weather Conditions and Maturity Group Impacts on the Infestation of First Generation European Corn Borers in Maize Hybrids in Croatia

Renata Bažok [1], Ivan Pejić [2], Maja Čačija [1,*], Helena Virić Gašparić [1], Darija Lemić [1], Zrinka Drmić [3] and Martina Kadoić Balaško [1]

[1] Department of Agricultural Zoology, University of Zagreb Faculty of Agriculture, Svetošimunska Cesta 25, 10000 Zagreb, Croatia; rbazok@agr.hr (R.B.); hviric@agr.hr (H.V.G.); dlemic@agr.hr (D.L.); mbalasko@agr.hr (M.K.B.)
[2] Department of Plant Breeding, Genetics and Biometrics, University of Zagreb Faculty of Agriculture, Svetošimunska Cesta 25, 10000 Zagreb, Croatia; ipejic@agr.hr
[3] Croatian Agency for Agriculture and Food, Plant Protection Center, Vinkovačka Cesta 63c, 31000 Osijek, Croatia; zrinka.drmic@hapih.hr
* Correspondence: mcacija@agr.hr; Tel.: +385-1-2393621

Received: 9 September 2020; Accepted: 16 October 2020; Published: 18 October 2020

Abstract: Overwintering success and weather conditions are the key factors determining the abundance and intensity of the attack of the first generation of European corn borers (ECB). The tolerance of maize to the 1st generation of ECB infestation is often considered to be connected with the maize maturity time. The aims of this research were (I) to examine the reactions of different maize FAO maturity groups in term of the damage caused by ECB larvae, (II) to analyze the influence of four climatic regions of Croatia regarding the damage caused by ECB larvae, and (III) to correlate observed damage between FAO maturity groups and weather conditions. First ECB generation damage has been studied in the two-year field trial with 32 different hybrids divided into four FAO maturity groups (eight per group) located at four locations with different climatic conditions. The results showed a lack of correlation between the FAO maturity group and the percent of damage. The percent of damage was positively correlated with the average air temperature in June (r = 0.59 for 2017 and r = 0.74 in 2018, $p = 0.0001$) within the range from 20 to 24.5 °C and was negatively correlated with the relative air humidity (r = −0.58 in 2017 and r = −0.77 in 2018, $p = 0.0001$) within the range of 50% to 80%. Our results provide a better understanding of the different factors that influence ECB damage. The obtained data could be used to predict the damage from the first generation of ECB under the weather conditions of different regions.

Keywords: *Ostrinia nubilalis*; tolerance; correlation; *Zea mays*; FAO maturity groups; damage

1. Introduction

The European corn borer (ECB) (*Ostrinia nubilalis* Hübn.), is a serious pest of maize (*Zea mays* L.) in Europe and the United States (US) as well. The pest is native to Europe [1] and was accidentally introduced in the US in 1917 and spread around the country causing serious damage. The losses are estimated at $1 billion per year [2]; hence, ECB is one of the most important pests from an economic point of view. The preferred host plant of this pest is maize [3]; however, ECB is a polyphagous insect and attacks many different plants, including sorghum, pepper, hemp, millet, chrysanthemums, and some weeds [4].

The extent of losses in maize caused by this pest depend on the degree of the infestation, the year, the yield averages and can range from 250–1000 kg/ha [3]. The damage is caused by the larvae that bore

into the stems and ears of corn [5]. Leaf feeding and stem tunneling by ECB larvae reduce plant growth and cause stalk lodging and ear dropping, resulting in severe yield losses of up to 30% [6]. Infestation by the first generation of ECB can reduce silage yields by 14% [7]. As a result of the damage, plants become more susceptible to secondary infections caused by different pathogens, such as *Fusarium* spp. or *Ustilago maydis* (DC.) Corda [8–10]. This is why Hudon et al. [11] suggested that the pest should be controlled when 40%–50% of the plants have been attacked.

According to Lynch et al., [12] many authors studied yield losses as a result of the damage caused by this pest. They concluded that several different things affect yield losses: the time of infestation, the stage of plant development when the infestation was initiated, and the geographical location. The first research in Croatia conducted by Ivezić [13] showed an average infestation rate of 37% by ECB. During the 1990s, many farmlands in the eastern part of Croatia were abandoned or neglected. Maize stalks were left in the field, which allowed the pest to spread and reproduce without interruption. Consequently, the damages and yield losses have increased. Yield loss in Croatia caused by the ECB range from 2% up to 25% [14]. Research from Ivezić and Raspudić [15] demonstrated that the average infestation rate during the five-year investigation (1992–1996) was 64%. Another research from Raspudić et al. [16] showed that the pest attacked up to 90% of the growing maize in Croatia. These numbers change depending on the climatic conditions, which have an impact on the insect's growth and development each year.

Many biotic and abiotic factors influence the appearance and intensity of an ECB attack; however, the weather conditions are the most significant ones [17]. The number of generations per year is connected with the climatic conditions. In contrast to the US Corn Belt, where the ECB has up to four generations per year, only one generation is observed in Central Europe [18]. According to Raspudić et al. [19], ECB has two generations in eastern Croatia per year. Daily temperatures and precipitation are very important factors for the ECB population dynamics [20]. We can expect greater damage to maize caused by ECB in a year with increased air temperatures and average precipitation [20].

Moths hatch during May and deposit their eggs on plants at the late whorl stage, before anthesis. The most sensitive stage of this pest to weather conditions is during the egg-laying stage, larva eclosion, and larva first instars. The minimum required temperature for ECB larvae development is 11 °C [21]. According to Rosca and Rada [22], moderate air temperature and high air humidity can result in increased larva eclosion and lower mortality of the first instar larva. On the other hand, high temperatures and drought resulted in higher mortality of ECB first instars larva and low larva eclosion [23]. Heavy storms registered in the period of larvae eclosion can have a negative effect on the population dynamics [24].

Overwintering success together with climatic conditions are the key factors determining the abundance and intensity of the attack of the first generation [25]. Lemić et al. [25], in their research, estimated that 8000 moths/ha can overwinter if a corn field is left unploughed. If one female moth can lay approximately 500 eggs [26] that results in 4 million larvae of the first generation. Thus, destroying severed maize stalks, where the ECB overwinters, is the most important mechanical measure and must be applied to the whole area where maize is grown. Control strategies for reducing yield losses from this insect include planting dates, early harvest, field scouting, using economic thresholds, insecticides, and hybrid resistance or tolerance.

Tolerance is the ability of a maize plant to withstand a certain population density of the insect without economic loss of yield or quality [27]. Yield losses would be much higher if modern maize hybrids did not have some degree of resistance to ECB [28]. Resilience to the ECB of the commercial maize hybrids is now a common feature. Approximately 90% of the 400 maize hybrids on the market have shown a certain degree of resistance in the vegetative phases of development [29]. Alongside resistance, modern maize hybrids are tolerant of a great degree of damage caused by ECB. Hybrid resistance to whorl feeding borers and tolerance to stalk and ear shank tunneling has increased dramatically from 1940s hybrids [30], with some seed companies providing first and second generation

corn borer tolerance ratings for their hybrids [31]. The development of tolerant maize hybrids with a strong, robust stalk contributes immensely to reducing yield loss as a consequence of the damage caused by the ECB [32].

Augustinović et al. [33], in their research, recorded differences between Croatian maize hybrids to ECB larvae feeding. ECB larvae prefer to feed on susceptible hybrids and they gain significantly more weight than larvae fed on tolerant hybrids [34]. Additionally, the tolerance to the first generation ECB infestation is very often connected with the maize FAO maturity group. Higher FAO groups of maize have intensive vegetative growth and, hence, a high and robust stems with a large number of big leaves. This is a biological characteristic that attracts the first generation of ECB, and thus, they lay more eggs. A high population level of the first generation could lead to a high level of second ECB generation, which can cause a yield reduction in the hybrids with longer vegetation periods (medium-late FAO maturity groups) [25].

FAO maturity groups may differ in their sensitivity to the first generation of ECB [25,33]. However, the sensitivity is also correlated with weather conditions, in particular with the average daily temperatures, relative air humidity, and the total amount of rainfall in May and June when egg laying and hatching and larval development is expected. Therefore, the aim of this research was to (I) determine the differences among FAO maturity groups regarding the damage caused by ECB larvae, (II) to establish the differences among four climatic regions of Croatia regarding the damage caused by ECB larvae, and (III) to investigate the correlation between the FAO maturity group and climatic factors.

2. Results

Statistical analysis of the weather conditions recorded in May and June showed that the temperatures in May and June significantly differed between years (Table 1). The average monthly temperature in May was lower in 2017 compared to 2018, while in June the average monthly temperature was higher in 2017 compared to 2018.

Table 1. Comparison of the weather conditions (the average monthly temperature in °C, total amount of rainfall in mm, and average air humidity in %) in May and June between 2017 and 2018 and the result of statistical analysis.

Weather Indicator	Average Value ± SD for Year [1]		$HSD_{p=0.05}$
	2017	2018	
Average monthly temperature in May (°C)	17.78 ± 0.83 b [2]	19.45 ± 0.83 a	1.5
Average monthly temperature in June (°C)	22.90 ± 1.02 a	21.55 ± 1.49 b	0.752
Total monthly amount of rainfall in May (mm)	58.48 ± 12.95	103.60 ± 40.21	ns [3]
Total monthly amount of rainfall in June (mm)	45.45 ± 24.99	118.20 ± 52.16	ns
Average air humidity in May (%)	68.00 ± 4.08	71.93 ± 5.91	ns
Average air humidity in June (%)	65.00 ± 5.66	70.23 ± 11.53	ns

[1] Means and SD values are shown in original data units. [2] Means followed by the same letter in the same row are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test). [3] ns—not significant at $p = 0.05$.

Out of the six weather indicators observed, two of them, the average monthly temperatures in June and the total amount of rainfall in June differed among the locations (Table 2). Šašinovec and Gola were locations with lower temperatures compared to Vrana, and, at the same time, Šašinovec and Tovarnik were locations with higher total amounts of rainfall compared to Vrana.

Table 2. Comparison among the locations in weather conditions (the average monthly temperature in °C, total amount of rainfall in mm, and average air humidity in %) in May and June and the result of statistical analysis.

Weather Indicator	Average Value ± SD for Location [1]				$HSD_{p = 0.05}$
	Šašinovec	Tovarnik	Gola	Vrana	
Average monthly temperature in May (°C)	18.10 ± 0.71	19.10 ± 1.98	17.95 ± 1.48	19.30 ± 0.57	ns [2]
Average monthly temperature in June (°C)	21.45 ± 1.20 b [3]	22.35 ± 0.92 ab	21.15 ± 1.20 b	23.95 ± 0.49 a	1.613
Total monthly amount of rainfall in May (mm)	98.60 ± 80.75	80.15 ± 30.05	65.30 ± 10.89	80.10 ± 27.72	ns
Total monthly amount of rainfall in June (mm)	108.25 ± 69.65	99.65 ± 91.57	83.90 ± 16.83	35.50 ± 27.72	ns
Average air humidity in May (%)	75.00 ± 2.83	65.75 ± 3.18	71.35 ± 4.74	67.75 ± 6.72	ns
Average air humidity in June (%)	75.20 ± 3.11	65.85 ± 6.86	72.00 ± 9.90	57.40 ± 5.09	ns

[1] Means and SD values are shown in original data units. [2] ns—not significant at $p = 0.05$. [3] Means followed by the same letter in the same row are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test).

Although the statistical differences among FAO maturity groups exist for both years of investigation (Table 3), we did not establish any correlation between the FAO maturity group and the percent of the damage caused by the first generation. The correlation coefficients were not significant in both years of investigation ($p = 0.6561$ in 2017 and $p = 0.3643$ in 2018).

Table 3. The average percent of plants (±SD) infested by European corn borer (ECB) larvae established on corn hybrids belonging to different FAO maturity groups in 2017 and 2018 and the results of statistical analysis.

FAO Maturity Group	2017	2018
FAO 300	14.13 ± 14.28 b [1]	20.57 ± 20.89 ab
FAO 400	15.57 ± 14.65 b	18.95 ± 18.03 b
FAO 500	18.46 ± 16.77 ab	23.54 ± 22.00 a
FAO 600	20.27 ± 20.31 a	23.71 ± 22.94 a
$HSD_{p=0.05}$ [2]	4.525	4.487

[1] Means and SD values are shown in original data units. [2] Means followed by the same letter within a column are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test).

The percentage of infestation of each of four FAO groups significantly differs among locations in both years of investigation (Tables 4 and 5). At the same time, the percentage of infestation significantly differs among FAO groups only once in each year (at only one locality in 2017 and at one locality in 2018).

Table 4. The average percent of plants (±SD) infested by European Corn Borer larvae established at four different locations and in four different regions in Croatia in 2017 and the results of the statistical analysis.

Locality	FAO Maturity Group				$HSD_{p=0.05}$ [3]
	300 [1]	400 [1]	500 [1]	600 [1]	
Šašinovec	1.54 ± 0.89 d [2] B [3]	1.6 ± 0.97 d AB	3.76 ± 1.00 b A	1.99 ± 1.08 c AB	2.21
Gola	6.29 ± 1.73 c	8.14 ± 1.74 c	7.93 ± 1.52 b	9.87 ± 1.99 b	ns [4]
Tovarnik	15.83 ± 1.55 b	17.43 ± 1.74 b	23.50 ± 1.47 a	23.58 ± 1.79 a	ns
Vrana	24.61 ± 1.50 a	27.02 ± 1.16 a	30.52 ± 1.71 a	34.47 ± 1.80 a	ns
$HSD_{p=0.05}$ [2]	3.44	3.69	4.54	4.99	

[1] Data were transformed by using *arc.syn x* transformation. Means and SD values are reported in transformed data units and are not de-transformed. [2] Means followed by the same small letter within the columns are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test); small letters refer to differences among locations. [3] Means followed by the same capital letter within the rows are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test); capital letters refer to differences among hybrids. [4] Not significant.

Table 5. The average percent of plants (±SD) infested by European corn borer larvae established at four different locations and in four different regions in Croatia in 2018 and the results of the statistical analysis.

Locality	FAO Group				$HSD_{p=0.05}$ [3]
	300 [1]	400 [1]	500 [1]	600 [1]	
Šašinovec	6.33 ± 1.17 c	8.56 ± 1.04 c	8.39 ± 0.95 c	8.78 ± 1.15 c	ns [4]
Gola	1.35 ± 1.00 d	1.68 ± 1.09 d	2.21 ± 1.09 d	1.36 ± 1.20 d	ns
Tovarnik	27.03 ± 1.95 b	25.03 ± 1.69 b	31.21 ± 1.84 b	30.22 ± 1.66 b	ns
Vrana	39.97 ± 1.29 a AB	33.97 ± 1.27 a B	45.59 ± 1.22 a A	47.14 ± 1.43 a A	9.85
$HSD_{p=0.05}$ [2]	3.36	3.05	3.50	3.26	

[1] Data were transformed by using *arc.syn x* transformation. Means and SD values are reported in transformed data units and are not de-transformed. [2] Means followed by the same small letter within the columns are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test): small letters refer to differences among locations. [3] Means followed by the same capital letter within the rows are not significantly different ($p = 0.05$; Tukey's honestly significant difference (HSD) test); capital letters refer to differences among hybrids. [4] Not significant.

The highest correlation coefficients (measured by Pearson's coefficient of correlation) and the highest coefficients of determination (Table 6) were obtained when the percent of infestation was correlated with the mean air temperature and with the average air humidity in June in both years of investigation. According to Roemer-Orphal, established correlations could be described as strong (for the mean air temperature in June in 2017 and 2018 and for the average air humidity in June 2017) or as very strong (for the average air humidity in June 2018). Although the percent of infestation significantly correlated with the total amount of rainfall in June in 2017 and in 2018 ($r = -0.5742$ and $r = -0.2582$, respectively), the correlation could be described as strong only in 2017. The amount of variability measured by the coefficient of determination (r^2) was higher for the average air temperatures and average air humidity in June 2018, with $r^2 = 0.557$ for the average air temperature and $r^2 = 0.6027$ for the average air humidity) than for June 2017 ($r^2 = 0.3563$ for the average air temperature and $r^2 = 0.3392$ for the average air humidity) confirming that the weather conditions in 2018 were more favorable for ECB development than in 2017.

Table 6. The correlation coefficients and coefficients of determination for ECB infestation expressed as a % of the attack of first generation as a dependent variable on different weather conditions (the mean air temperature, total amount of rainfall, and average air humidity) as independent variables in two years of investigation.

Independent Variable	Month	Year	*n*	Correlation Coefficient r	Coefficient of Determination r^2	*p*	Type of Correlation
Mean air temperature	May	2017	512	0.48	0.2375	0.0001	medium
	May	2018	512	0.57	0.3274	0.0001	strong
	June	2017	512	0.59	0.3563	0.0001	strong
	June	2018	512	0.74	0.5570	0.0001	strong
Total amount of rainfall	May	2017	512	0.23	0.0574	0.0001	very weak
	May	2018	512	0.03	0.0014	0.0405	not existing
	June	2017	512	−0.57	0.3298	0.0001	strong
	June	2018	512	−0.25	0.0667	0.0001	weak
Average air humidity	May	2017	512	−0.59	0.3567	0.0001	strong
	May	2018	512	0.14	0.0225	0.0007	very weak
	June	2017	512	−0.58	0.3392	0.0001	strong
	June	2018	512	−0.77	0.6027	0.0001	very strong

The regression analysis performed for average monthly temperature in June (Figure 1) show that there is a linear growth in the percent of plants infested by the first generation of ECB larvae with the increase of average air temperatures in June from 20 °C to 24.5 °C.

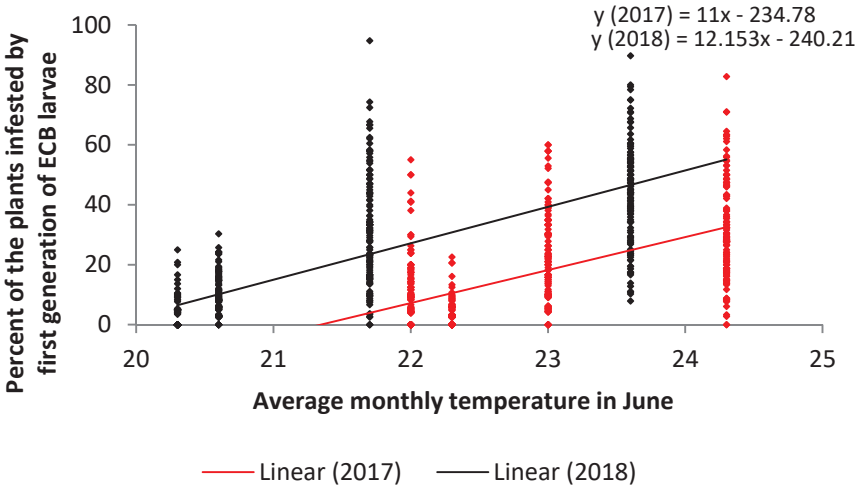

Figure 1. Regression analysis of the average monthly temperature in June (x) versus the percent of infestation with first generation of ECB larvae (y) in two years (2017—red and 2018—black).

The regression analysis performed for average air humidity in June (Figure 2) shows that there is a linear decrease in the percent of plants infested by the first generation of ECB larvae along with the increase of average air humidity in June from 50% to 80% relative air humidity.

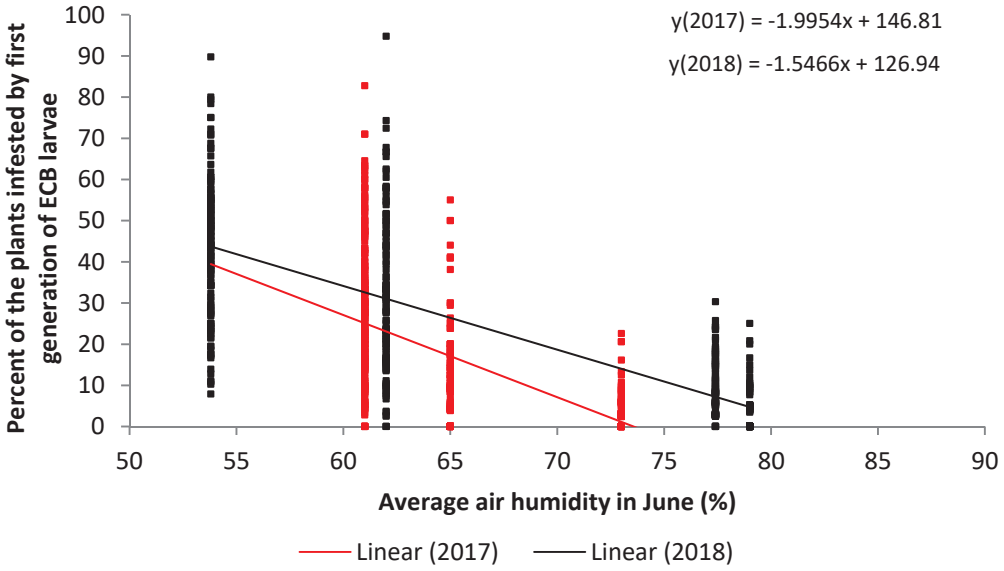

Figure 2. Regression analysis of the average air humidity in June (x) versus the percent of infestation with first generation of ECB larvae (y) in two years (2017—red and 2018—black).

3. Discussion

In the literature review, opposing data on the tolerance of different FAO maturity groups to ECB can be found. The tolerance is correlated with the agronomic and morphological traits of different FAO maturity groups rather than with any mechanism of the tolerance [30]. For example, Patch [35] reported that the height of maize or a factor, such as maturity correlated with height, was the main factor in the selection of maize by the ECB moths for oviposition. Maize hybrids planted earlier and the hybrids with extensive vegetative growth were attractive to moths to lay eggs, and therefore those hybrids suffered higher infestation from first generation ECB [25,32]. Recent investigations conducted by Leppik and Frérot [36] reported on maize odorscapes under field conditions that may improve host plant detection in ECB moths during oviposition.

To the best of our knowledge, this is the most comprehensive investigation of the Croatian market maize hybrids and the possible difference in their tolerance to ECB infestation. The insect pest resistance of hybrids on market is typically not declared. Thus, the research of tolerance to certain pests is a target of the research as is the case with Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) not only in Croatia [37], but also in other neighboring countries [38] and at the general scale [39].

The results with Croatian hybrids and their tolerance to ECB reported by Ivezić and Raspudić [15], Lemić et al. [25] and Augustinović et al. [33] were based either on smaller number of hybrids or on the few locations included in the investigation. The lack of correlation between the FAO maturity group and the percent of infestation obtained in our study was in line with the data reported by Augustinović et al. [33], who reported significant differences in the intensity of the damaging effects on different locations and no significant differences concerning various hybrids. Similar results were obtained in the study with commercial maize hybrids in Poland [40], as well as with sweet corn [41] where it was shown that the percentage damage of the ECB larvae was different in each of the sampling plots and variety. In addition, the ECB larval damages were different for each of the sweet corn varieties, proving that the damage could not be correlated with hybrid, proving that locality and year had a major impact on the ECB attack.

The differences in the percent of attacked plants among hybrids have been established in the trials carried out at Šašinovec in 2017 and in Vrana in 2018 (Tables 4 and 5). Based on the obtained results we cannot confirm that the strong and robust stem hybrids belonging to the later maturity group are more tolerant and do not suffer significant yield loss, in spite of the significant damage as reported by Lemić et al. [25] and Raspudić et al. [32]. In our study, we did not investigate the second generation attack and the yield loss; therefore, we cannot conclude on the tolerance to the yield loss.

Weather conditions were listed by many authors as the most significant factor influencing the appearance and intensity of ECB attacks [17,20,22,23,25]. Eclosion of the moths in Croatian conditions [14] is expected in May and egg laying occurs in May and in June, while egg hatching and larval development is expected in June. Therefore, we assumed that the weather conditions in May and June would be the critical for the first generation attack. Comparing the two years in which our investigation was performed, we observed that a significant difference was established between the years in the average air temperature in May and June.

The higher temperature was recorded in 2018 compared to 2017. Contrary to that, the average air temperature in June was higher in 2017 compared to 2018 (Table 1). Generally, in 2018, a higher total amount of rainfall was recorded compared to 2017; however, the difference between the years was not significant (Table 1). Among the locations, significant differences were established in the average air temperature in June and in the total monthly amount of rainfall in June (Table 2). The observed differences in weather conditions allow us to make conclusions regarding their impact on ECB attacks.

Many authors agree that weather conditions greatly influence ECB populations [42–44]. Rosca and Rada [22] reported on the positive impact of moderate air temperature and high air humidity on egg hatching and larval development. Barbulescou et al. [23] reported on the negative impact of high temperatures and drought resulting in high ECB larval mortality. The amount of precipitation in May and June and average air humidity in May and June were higher in 2018, comparing to 2017. Even though the differences were not significant, we can conclude that year 2018 was more favorable for ECB development than year 2017. This is confirmed by our results (Table 3).

The percent of plants infested on a single FAO hybrid at particular location (Tables 4 and 5) ranged from 1.54% to 34.47% in 2017 and from 1.35% to 47.14% in 2018, respectively. The difference in the percent of plant infestation of all investigated maturity groups was established among locations in both years of investigation proving that weather conditions have major influence on the intensity of attack. Maize hybrids planted on locations in the mid part of Croatia, Šašinovec, and Gola, where the temperatures were lower and the amounts of rainfall were on average (but higher comparing to Vrana), recorded lower damages compared to Vrana, measuring higher temperatures in June in both years.

Intensive vegetative growth is a biological characteristic that attracts the first generation of ECB to intensifying their egg laying. However, the weather conditions are a crucial factor influencing the moth activity in June, as well as the egg laying, and egg hatching. Our results confirmed that the first generation attack of ECB was correlated with the weather conditions in June while the weather conditions in May were of less importance (Table 6). This is likely due to the fact that in Croatian conditions, oviposition and egg hatching took place in June [45].

According to many authors [42–44], oviposition and larval survival were reduced in years in which the temperatures or precipitations were below the average during the oviposition period. When the temperatures and precipitation were normal or above the average during oviposition, more ECB eggs were laid and the larval survival was higher. This was partially confirmed by our results as we established a strong to very strong correlation between average monthly temperature in June and the percent of attack intensity.

The regression line was linear and positive, which indicates that the percent of infestation increased with the increase of the average monthly temperature in June from 20 °C and 24.5 °C (Figure 1). Contrary to the statements that normal and increased precipitations in the oviposition period have a positive impact on larva development, our results did not prove a consistent impact of the total amount of rainfall in May and June on an increase or decrease of the percent of ECB attack intensity. Our results confirmed a negative impact of the increase of average air humidity (which is indirectly influenced by the amount of rainfall) in June on the percent of attacks in both years of investigation.

The regression line was negative and showed that the air humidity over 75% could be critical for larval development (Figure 2). Data presented by Showers et al. [46] implicate moisture (including inundation) and evaporation as especially potent factors in the suppression of the first and second instars of first generation ECB. However, it is difficult to compare our results because their observations were done under much higher temperatures (between 25 °C and 31 °C), and the moisture was expressed as moisture loss.

For egg laying and egg hatching, a warm and medium dry June is favorable. A high population level of the first generation, as we observed at Vrana and Tovarnik, may lead to a high level of second ECB generation as was reported by Lemić et al. [25], which ultimately caused yield reduction. In our investigation, we did not evaluate the attack of the second generation and did not compare the yield among hybrids. In the future, it would be interesting to evaluate the second generation as this can significantly increase the yield loss. However, establishing the yield loss and comparison among hybrids would be possible only between the same hybrids (untreated and treated with the complete protection against ECB).

4. Materials and Methods

4.1. Experimental Fields and Trial Design

Research was conducted in 2017 and 2018 at four locations in different climatic regions of Croatia: Šašinovečki Lug (45°51′00″ N, 16°10′01″ E; Central Croatia), Gola (46°1′44″ N, 16°33′13″ E; North-West Croatia), Tovarnik (45°13′28″ N, 19°21′38″ E; East Croatia), and Vrana (43°56′45″ N, 15°26′53″ E; Adriatic coast). Depending on the location, from 11 April until 5 May in 2017 and from 15 April until 6 May in 2018, in each of the four locations, 28 maize hybrids of Croatian breeding companies and four international hybrids belonging to four FAO maturity groups (300, 400, 500, and 600) were sown by row-column design in four replications. Every FAO maturity group was represented by eight commercially available hybrids (Table 7). In each group, one international hybrid and seven nationally developed and widely sown hybrids have been included. The hybrids were planted in four replication on 10 m^2 plots (four 3.57 m long rows at row distance 0.7 m) with appropriate plant density.

Table 7. List of the maize hybrids involved in the investigation.

FAO 300		FAO 400		FAO 500		FAO 600	
Hybrid	**Company**	**Hybrid**	**Company**	**Hybrid**	**Company**	**Hybrid**	**Company**
Bc 344	Bc [1]	Os 444	Os	Os 552	Os	Bc 682	Bc
Bc 323	Bc	Bc 406	Bc	Bc 525	Bc	Bc 616	Bc
Bc 306	Bc	Bc 424	Bc	Bc 575	Bc	Bc 626	Bc
TRIO	Bc	Bc 482	Bc	Klipan	Bc	Riđan	Bc
P9903	DuPont [2]	DKC 4608	DeKalb [4]	DKC 5830	DeKalb	P1535	DuPont
Os 378	Os [3]	Kulak	Os	Velimir	Os	Rudolf 60	Os
Os 398	Os	Tomasov	Os	Os 5922	Os	Os 6217	Os
Os 3617	Os	Drava 404	Os	Os 515	Os	Os 635	Os

[1] Bc—Bc Institute Rugvica, Dugoselska 7, Croatia. [2] DuPont—Corteva Agroscience, Chestnut Run Plaza 735, Wilmington, DE, 19805-0735, USA. [3] Os Agricultural Institute Osijek, Južno predgrađe 17, Osijek, Croatia. [4] DeKalb—DeKalb Genetics Corporation, 3100 Sycamore Rd, DeKalb, IL 60,115 United States.

4.2. Meteorological Data

Data collection on the average daily air temperature, daily amount of rainfall, and relative air humidity was done by setting up an automatic weather station (Davis 6250EU, Davis Instruments, Hayward, CA, USA) in the period between the first of May and the 30th of June in both years next to the maize fields at each location (Šašinovečki Lug, Gola, Tovarnik and Vrana).

4.3. Trial Assessments

The intensity of the first ECB generation attack was recorded between 28 June and 17 July 2017, as well as between 19 June and 11 July 2018. Within a plot all plants in the two inner rows of every replication, (i.e., 35–40 plants per replication or 160 plants per hybrid) were inspected on each location. The number of inspected and the number of damaged plants were recorded., only the number of Distinctive leaf holes and shot holes on stalks were identified as damage on the plants. The severity of symptoms was not recorded. The percent of attacked plants was calculated as a ratio of the number of attacked and the number of inspected plants.

4.4. Data Analysis

All data on the percent of infestation were compared between the FAO maturity groups, regions, and years by ANOVA by statistical software ARM 9® [47], and the mean separation was estimated using Tukey's honestly significant difference (HSD) test.

When required to correct skewness, the data were transformed using the *arc.syn x* or $\sqrt{x + 5}$ transformation. Statistical software ARM 9® [47] was used to calculate the correlation coefficients and to conduct regression analyses between the mean monthly air temperature, the total monthly amount of rainfall, and the average monthly air humidity as independent variables, and the percent of infestation as a dependent variable. The Pearson's correlation coefficients were established, regression lines were described, and the coefficient of determination was calculated.

5. Conclusions

While intensive vegetative growth (often associated with hybrids belonging to later maturity groups) may attract ECB moths to lay eggs, and influence the attack of the first generation of ECB, the first generation attack was found to be primarily influenced by the weather conditions during the period of egg laying and hatching as well as during larval development. The first generation attack was positively correlated with the average air temperature in June within the range of 20 to 24.5 °C and was negatively correlated with the relative air humidity within the range of 50% to 80%. Our results provide a better understanding of the different factors influencing ECB damage. The obtained

results could be useful for prediction of the damage from the first generation of ECB under the weather conditions similar to those observed in this research.

Author Contributions: Conceptualization, R.B., I.P., M.Č. and M.K.B.; Data curation, R.B.; Formal analysis, R.B. and Z.D.; Funding acquisition, R.B.; Investigation, M.Č., H.V.G., D.L., Z.D. and M.K.B.; Methodology, R.B., M.Č. and Z.D.; Project administration, M.Č.; Resources, R.B. and I.P.; Software, R.B.; Validation, R.B. and D.L.; Writing—original draft, R.B., M.Č. and M.K.B.; Writing—review & editing, I.P. and D.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by the Croatian Science Foundation trough projects: AGRO-DROUGHT-ADAPT (2016-06-8290) "Adaptability assessment of maize and soybean cultivars of Croatia in the function of breeding for drought tolerance", MONPERES (2016-06-7458) "Monitoring of Insect Pest Resistance: Novel Approach for Detection, and Effective Resistance Management Strategies" and Young researchers' career development project—training of new doctoral students (DOK-01-2018). Publication was supported by the Open Access Publication Fund of the University of Zagreb Faculty of Agriculture.

Acknowledgments: The authors thank all collaborators on the projects, coauthors on publications, colleagues, students, and technical staff who contributed to field work as well as the anonymous reviewers.

Conflicts of Interest: The authors declare no conflict of interest.

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MDPI AG
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ISBN 978-3-7258-5274-1