

1 Article

2 SpitWorm, an herbivorous robot: Mechanical leaf 3 wounding with simultaneous application of salivary 4 components

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16 **Abstract:** Induction of jasmonate-mediated plant defense against insect herbivory is initiated by a
17 combination of both, mechanical wounding and chemical factors. In order to study both effects
18 independently on plant defense induction, SpitWorm, a computer-controlled device which mimics
19 the damage pattern of feeding insect larvae on leaves and, in addition, can apply oral secretions (OS)
20 or other solutions to the ‘biting site’ during ‘feeding’, was developed and evaluated. The amount of
21 OS left by a *Spodoptera littoralis* larva during feeding on *Phaseolus lunatus* (lima bean) leaves was
22 estimated by combining larval foregut volume, biting rate, and quantification of a fluorescent dye
23 injected into the larvae’s foregut prior to feeding. For providing OS amounts by SpitWorm equivalent
24 to larval feeding, dilution and delivery rate were optimized. The effectiveness of SpitWorm was tested
25 by comparing volatile organic compounds (VOC) emissions of *P. lunatus* leaves treated with either
26 SpitWorm, MecWorm or *S. littoralis* larvae. Identification and quantification of emitted VOCs revealed
27 that SpitWorm induced a volatile bouquet that is qualitatively and quantitatively similar to herbivory.
28 Additionally, RT-qPCR of four jasmonic acid responsive genes showed that SpitWorm, in contrast to
29 MecWorm, induces the same regulation pattern as insect feeding. Thus, SpitWorm mimics insect
30 herbivory almost identical to real larvae feeding.

31 **Keywords:** Plant defense; Herbivory; Mechanical wounding; Oral secretions; Induced volatiles;
32 SpitWorm; MecWorm; *Phaseolus lunatus*; *Spodoptera littoralis*; Volatile organic compounds

33 1. Introduction

34 Standing at the beginning of the food chain, plants undergo biotic and abiotic challenges from the
35 environment. In nature, herbivorous insects are one of their major threats, especially in higher plants.
36 Despite their physical immobility, plants have survived and propagated for hundreds of millions of
37 years. During this long time, they have coevolved with herbivorous insects and developed strategies
38 to fend, repel and annihilate their insect enemies [1]. Plant defense strategies against herbivores have
39 aroused ardent and tremendous interests and research with profound achievements, especially in the
40 last 30 years [2]. These studies have deciphered that the feeding of insects can initiate a series of diverse
41 defense related events *in planta* such as signaling processes, jasmonate accumulation, specific gene and
42 protein expression patterns, and the production and accumulation of secondary metabolites including
43 volatile emissions [3]. Besides the wounding trauma, all the defense responses of plant are triggered by
44 herbivory associated molecular patterns (HAMPs) [4-7] but our understanding of these HAMPs is still
45 in the very early stages. To date, HAMPs can be classified into two categories: (i) chemical elicitors
46 derived from herbivore oral secretions, oviposition fluids or environmental DNAs (eDNA) that were
47 left behind by insects; and (ii) those that originate from the specific patterns of wounding, i.e. the

48 mechanical damage and the resulting elicitors from plants. This second category is also called damage
49 associated molecular patterns (DAMPs). Only both aspects together are able to induce the full spectrum
50 of plant herbivory defenses [8].

51 To study the contributions of the two aspects (mechanical wounding and chemical elicitors),
52 mechanical wounding of insect feeding was originally mimicked with different tools, including razor
53 blades [9-11], pattern wheels [12-14], forceps [15-17], paper punch [18] and needles [19]. However, using
54 lima bean (*Phaseolus lunatus*) as a model plant, mechanical wounding alone by cuts or scratches did not
55 induced volatile emission. Only by continuous mechanical wounding by a computer-controlled device
56 (MecWorm) which mimics the leaf wounding pattern of a feeding insect, caused an intense emission of
57 a blend of volatiles [8,20]. Those results indicated that mechanical wounding itself plays important roles
58 in plant defense induction. For a period of time, mechanical wounding with single or a few cuts or
59 scratches with different wounding tools was used as a control or in combination with larval oral
60 secretions (OS) or OS elicitors to study the defense inducing roles of chemical compounds from OS.
61 This was effective in inducing plant defense responses such as volatile emission and jasmonic acid (JA)
62 burst [21].

63 These elicitors include small molecular size fatty acid - amino acid conjugates (FACs) [21-24];
64 inceptins [25,26]; caeliferins [26] and volicitin [27]; glucose oxidase (GOX) [28] or a β -glucosidase [29]
65 as well as pore or channel forming compounds [30,31] were reported to induce signaling pathways,
66 biosynthesis of phytohormones and volatile emissions. However, compared with the vast diversity of
67 herbivores that attack plants, the known herbivore-derived elicitors are relatively few. Moreover, the
68 molecular mechanism of plant perception of these elicitors needs further study [32].

69 Methods to study insect OS or OS derived elicitors include mainly applying saliva or related
70 components onto wounds to mimic insect feeding and examine plant defense response. Up to now, no
71 standard procedure for wounding or OS application was established. Thus, besides different ways of
72 wounding itself, varying OS amounts applied (1 to 20 μ L), dilution factors up to 1:5, and differing
73 wounding areas, ranging from a few scratches or puncture rows up to 2% of the total leaf, can be found
74 in the various studies [12,14,33].

75 To examine the effect from different amounts and concentrations of insect OS applied to
76 mechanical wounding Musser, et al. [34] prevented the delivery of larval saliva (*Helicoverpa zea*) during
77 feeding. By using this technique, they showed that in tobacco plant defense responses to caterpillar
78 feeding were qualitatively different when caterpillars are either able or not able to secrete saliva. In
79 another case, Major and Constabel [13] used a dilution range from 1:1 to 1:180 to optimize the aqueous
80 dilution of OS from *Malacosoma disstria* applied to poplar leaves with over 100 puncture holes for
81 maximal target gene induction (*PtdTI3*). The reported quantitative effects of insect saliva introduced to
82 plant wounding area indicate that it is important to quantify the delivery ability of saliva from insect
83 to plant, i.e. how much saliva is delivered per bite by insect.

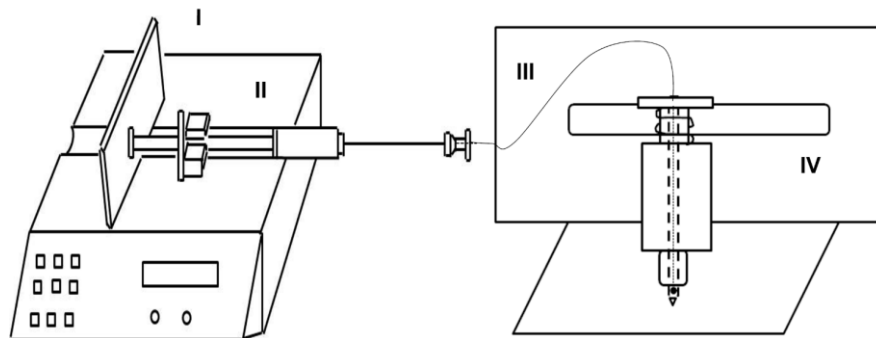
84 However, from all the former studies, one can estimate that the quantities of OS applied to
85 mechanical wounded plants were often several thousand times higher than the real amount left behind
86 at the wounding site by a larva per feeding bout, which was estimated in the range of 0.5 to 5 nL
87 (*Heliothis virescens* feeding on corn and tomato, respectively) [35]. To precisely mimic insect feeding, it
88 is necessary to determine the real amount left at the wounding zone by insect feeding.

89 Although the development of MecWorm provided deeper insight in our understanding of insect
90 herbivory, it was necessary to take the next step in order to mimic insect feeding as close as possible.
91 Thus an insect feeding-mimicking device was established that combines both mechanical wounding
92 and the simultaneous application of chemical elicitors. This so-called SpitWorm was engineered and
93 tested in comparison with both MecWorm and *S. littoralis* larvae feeding on induced defense responses
94 in lima bean.

95 2. Results

96 SpitWorm, based on the robotic system MecWorm [20], was developed by adding a syringe
97 connected to a capillary running through the inner-hollow of the 'biting' needle of MecWorm's punch

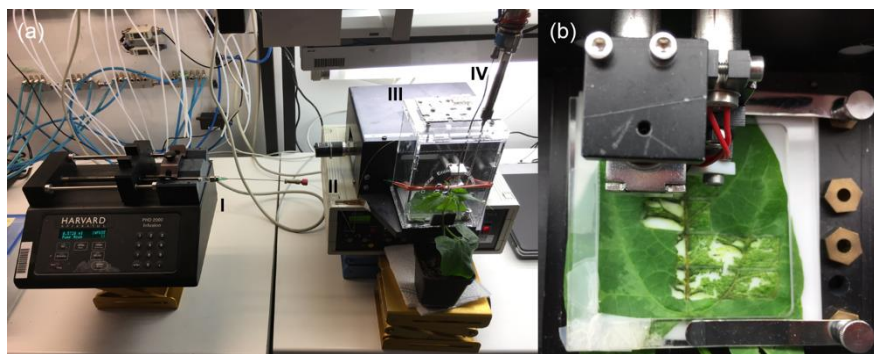
98 head until to a hole close to the needle tip. The syringe was actuated by a syringe pump to generate a
 99 stable and quantitative fluid delivery (Figure 1).



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101 **Figure 1.** Schematic sketch of SpitWorm. (I) Syringe pump controlling the fluid flow rate; (II) Syringe
 102 (100 μL) filled with diluted OS; (III) capillary from the syringe to the tip of MecWorm's hollow punching
 103 needle; (IV) MecWorm, a robotic system for continuous wounding.

104 For headspace volatiles collection of control and *S. littoralis* treated leaves as well as for MecWorm
 105 and SpitWorm treatments, the test leaves were enclosed in an acryl glass case together with the punch
 106 head and equipped with a closed-loop volatile collection pump system (Figure 2).



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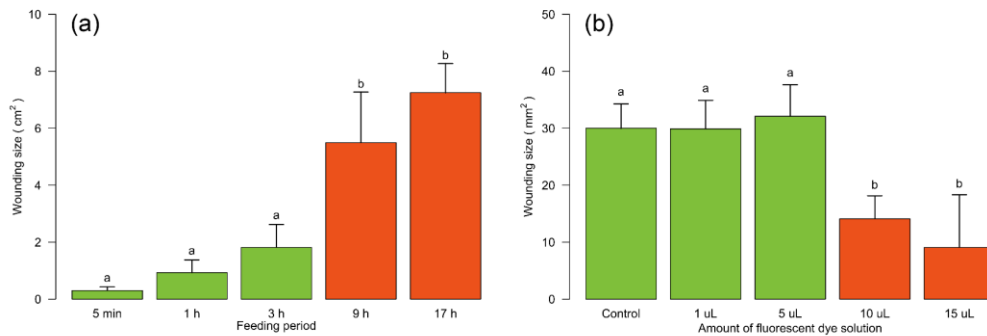
108 **Figure 2.** Volatile collection with SpitWorm. (a) MecWorm/SpitWorm. (I) automatic syringe pump with
 109 a 100 μL syringe; (II) capillary for OS delivering; (III) Plexiglas case; (IV) volatile collector. (b) Close-up
 110 of the biting head of SpitWorm punching a lima bean leaf. For MecWorm treatment, the capillary was
 111 removed and for larval treatment the system was switched off.

112 Results of the parameter evaluation like fluid delivery rate, dilution of OS of SpitWorm and the
 113 comparison of MecWorm, SpitWorm and *S. littoralis* treatment are described below. A schematic sketch
 114 of the several steps for determining the OS amount left on a leaf by *S. littoralis* can be found in the
 115 supporting information (Figure S1).

116 2.1. Wounding sizes of leaves fed by *S. littoralis* larvae

117 In order to adjust the wounding sizes to be generated by MecWorm and SpitWorm, leaf wounding
 118 sizes of different larval feeding periods were measured. With four replicates for each treatment, the
 119 mean wounding sizes upon larval feeding were after 5 min ($0.30 \pm 0.13 \text{ cm}^2$), 1 h ($0.93 \pm 0.45 \text{ cm}^2$), 3 h
 120 ($1.81 \pm 0.81 \text{ cm}^2$), 9 h ($5.49 \pm 1.78 \text{ cm}^2$), and 17 h ($7.25 \pm 1.02 \text{ cm}^2$), see Figure 3a.

121 Feeding activities of untreated larvae (control) were compared with the feeding performance of
 122 larvae injected with different volumes of fluorescent dye solution into their foregut to determine the
 123 optimal injection volume for the subsequent experiments (Figure 3b). Injection volumes of 1 μL and 5
 124 μL showed no significant differences (control mean, $29.98 \pm 4.29 \text{ cm}^2$; 1 μL mean, $29.88 \pm 5.02 \text{ cm}^2$; control
 125 $\sim 1 \mu\text{L}$, $p = 0.999$; 5 μL mean, $32.10 \pm 5.54 \text{ cm}^2$; control $\sim 5 \mu\text{L}$, $p = 0.978$) in leaf wounding sizes whereas
 126 injected volumes $\geq 10 \mu\text{L}$ led to a significant decrease in feeding activity (10 μL mean, $14.10 \pm 4.03 \text{ cm}^2$;
 127 15 μL mean, $9.08 \pm 9.26 \text{ cm}^2$; control $\sim 10 \mu\text{L}$, $p = 0.003$; control $\sim 15 \mu\text{L}$, $p = 0.0002$).



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Figure 3. Wounding sizes of lima bean leaves. (a) *S. littoralis* feeding for 5 min, 1 h, 3 h, 9 h and 17 h. (b) After 5 min feeding of *S. littoralis* injected with different volumes of fluorescence dye solution. Larvae not injected served as control. Mean \pm SD, $n = 4$, one-way ANOVA, post hoc test: Tukey's HSD, treatments with identical letters are not significantly different.

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Due to a stronger and clearer fluorescence signal at the wounding edges (Figure S2) an injection volume of 5 μ L of Lucifer Yellow solution was used in subsequent experiments.

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2.2. Residence time of fluorescent dye in the larval foregut

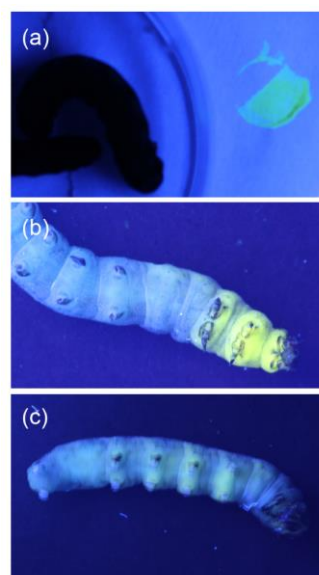
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Observation of fluorescent dye injected larvae under UV light showed that it takes 45 min to 1 hour for the fluorescence dye to start moving through the whole body of insect to the anus (Figure 4). Therefore, all experiments with fluorescent dye and insect dissection were conducted immediately or at least within 30 min after injection.



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Figure 4. Fluorescence images of a *S. littoralis* larva. (a) Larva before injection (on the right: a spot of Lucifer Yellow on filter paper), (b) 10 min, and (c) 1.5 h after injection of Lucifer Yellow solution (5 μ L, 1 $\text{mg}\cdot\text{mL}^{-1}$) into the larval foregut.

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2.3. Estimation of larval foregut volume

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Based on the relatively simple structure of the foregut of a *S. littoralis* larva, its shape was taken as cylinder. Measuring of dissected foreguts ($n = 5$) resulted in an average foregut length $l = 4.3 \pm 0.8$ mm; average width $d = 3.8 \pm 0.4$ mm resulting in an average foregut volume (V_g) of 49 ± 17.3 μ L, (Table S1).

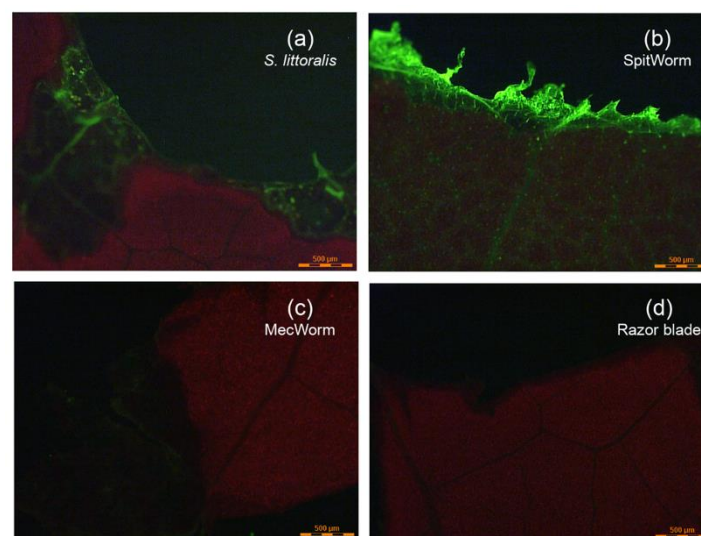
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2.4. Optimal flow rate for OS delivery in SpitWorm

149 The optimal flow rate for fluid delivery in SpitWorm was evaluated by visually comparing ink
 150 trails left on a filter paper during moving the punch head on a zigzag path (Figure S3d) and observing
 151 the needle tip of the punching head. As long as the delivery rate of the ink was sufficient to form a
 152 small droplet, a continuous trail of ink was obtained. At a delivery rate of $2.5 \text{ nL}\cdot\text{s}^{-1}$ the ink trace was
 153 weak and vanished after a short distance. With $5 \text{ nL}\cdot\text{s}^{-1}$ the trail got weaker during moving and
 154 vanished in the third line of the zigzag path. A delivery rate of $10 \text{ nL}\cdot\text{s}^{-1}$ which left an uninterrupted ink
 155 trail was chosen for subsequent SpitWorm experiments.

156 2.5. Fluorescent microscope images of different treatments

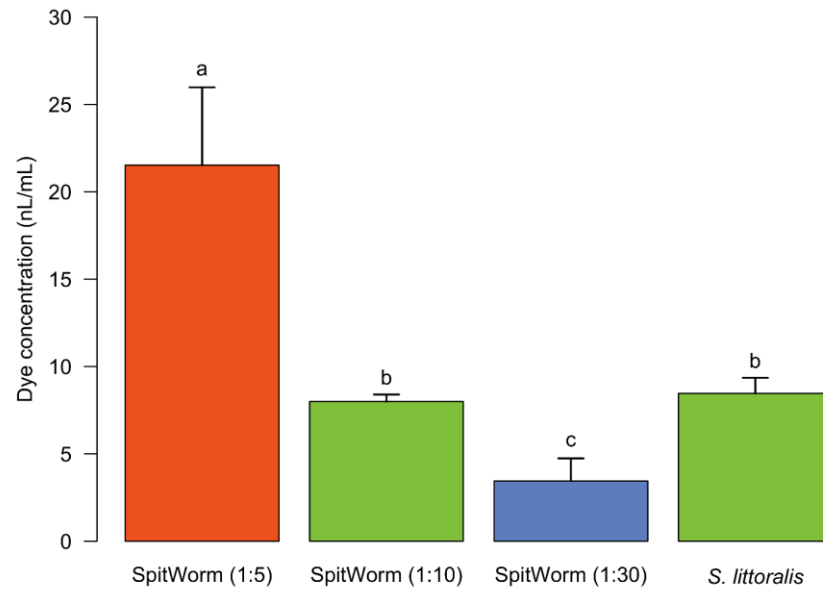
157 After treatment with a labeled larva (Figure 5a) and SpitWorm delivering labeled larval OS (Figure
 158 5b), respectively, a distinct fluorescence signal could be detected at the wounding edges of the leaves.
 159 After treatment with MecWorm (Figure 5c) and cutting with a razor blade (Figure 5d), respectively no
 160 fluorescence could be detected. The comparison of the OS trail left by larva and SpitWorm, respectively
 161 showed that the insect's OS went deeper into the vascular bundles of the leaf.



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 163 **Figure 5.** Fluorescent microscope images of lima bean leaves after different treatments. Detail view of
 164 wounding edges of the adaxial leaf surface. Treatments: (a) *S. littoralis* larva injected with Lucifer Yellow
 165 solution; (b) SpitWorm delivering Lucifer Yellow labeled larval OS; (c) MecWorm; and (d) cut with a
 166 razor blade.

167 2.6. Fluorescent dye quantification of tissues at the wounding edges after different treatments

168 In order to adjust the amount of OS delivered by SpitWorm, concentrations of Lucifer Yellow were
 169 measured after extraction of wounding edge tissues of leaves treated by fluorescent labeled larvae and
 170 compared with the concentrations in extracts of wounding edge tissues after treatments with
 171 SpitWorm, delivering different dilutions of labeled larval OS. As shown in Figure 6 clear differences of
 172 the extracted amounts of fluorescent dye between larval and SpitWorm treatment were observed for
 173 1:5 (mean \pm SD, $20.52 \pm 4.45 \text{ nL}\cdot\text{mL}^{-1}$, $p < 0.001$) and 1:30 (mean \pm SD, $3.45 \pm 1.29 \text{ nL}\cdot\text{mL}^{-1}$, $p = 0.032$)
 174 dilutions of labeled OS, respectively, whereas a dilution of 1:10 (mean \pm SD, $7.99 \pm 0.40 \text{ nL}\cdot\text{mL}^{-1}$, $p =$
 175 0.816) resulted in a concentration range close to larval treatment (mean \pm SD, $8.46 \pm 0.52 \text{ nL}\cdot\text{mL}^{-1}$)



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Figure 6. Fluorescent dye concentrations extracted from wounding edge tissues after different treatments. Treatments, *S. littoralis* injected with fluorescent solution; SpitWorm delivering different dilutions of labeled larval OS (1:5, 1:10, 1:30), $n = 3$ for each treatment, mean \pm SD, one-way ANOVA, post hoc test: Fisher's LSD, treatments with identical letters are not significantly different.

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2.7. Estimation of OS amount left by insect onto plant wounds per bite

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The observation of larvae that were feeding on lima bean leaves revealed a biting rate (BR) of 3-5 bites \cdot s $^{-1}$. Combining the BR with the mean foregut volume (V_g , 49 μ L), the amount of fluorescent dye solution injected (V_i , 5 μ L), the average concentration of OS left at the wounding edges by labeled larvae (C_d , 8.46 nL \cdot mL $^{-1}$), the feeding time (t , 300 s), and the solvent volume used for extraction of tissues from the wounding edges (V_s , 1 mL), the amount of OS left per bite (V_b) was calculated according to the following equation:

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$$V_b = \frac{C_d \times V_s \times \frac{V_g}{V_i}}{t \times BR}$$

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Taking the three different BR into account the following volumes of OS left at the wounding edges per bite were calculated: 3 bites \cdot s $^{-1}$, 92 pL \cdot bite $^{-1}$; 4 bites \cdot s $^{-1}$, 69 pL \cdot bite $^{-1}$; 5 bites \cdot s $^{-1}$, 55 pL \cdot bite $^{-1}$; mean \pm SD, 72 \pm 18.6 pL \cdot bite $^{-1}$.

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2.8. Volatile organic compounds released upon different treatments

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After optimizing the SpitWorm parameters, the next step was to test its abilities to provoke insect feeding like defenses. In the headspace of lima bean leaves treated with *S. littoralis* larvae, MecWorm and SpitWorm, respectively, 38 different compounds were identified and quantified relative to an internal standard (IS). Identified compounds, their retention indices, relative amounts are listed in Table 1 together with the number of replicates per treatment

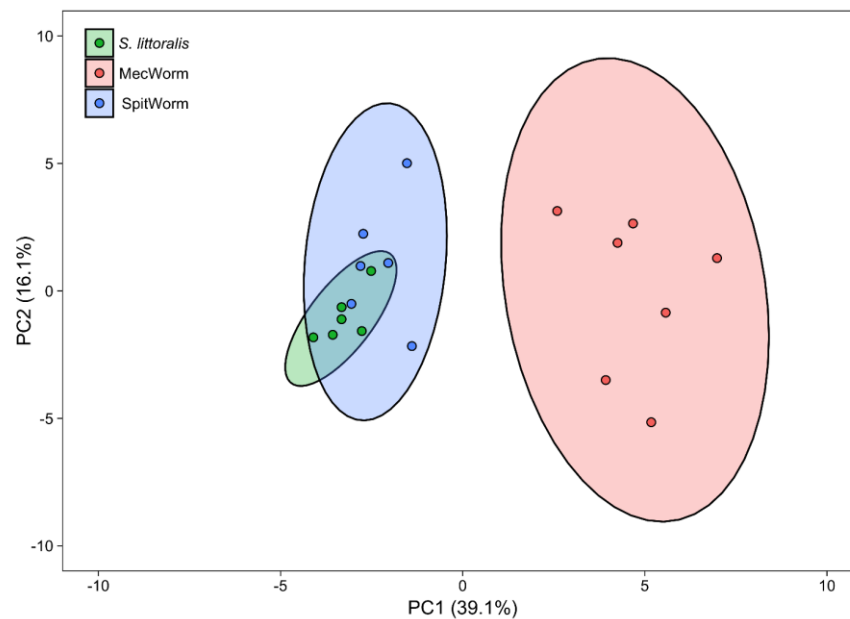
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Table 1. Analysis of volatile compounds identified and quantified in the headspace after different treatments. Retention indices: RI_{exp}: determined in this study; RI_{lit}: literature data from [NIST \[36\]](#) or [§ Adams \[37\]](#). Multiple comparisons for each compound (n.d., not detected) were performed by one-way ANOVA followed by Tukey's HSD post-hoc test; significance levels: n.s. ($p > 0.05$), * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Nr	Compound	Retention index		Relative amounts								p-values			Significance levels			
		RI _{exp}	RI _{lit}	<i>S. littoralis</i>		MecWorm		SpitWorm		Control		<i>S. littoralis</i> -	<i>S. littoralis</i> -	MecWorm -	<i>S. littoralis</i> -	<i>S. littoralis</i> -	MecWorm -	
				mean	±SD	mean	±SD	mean	±SD	mean	±SD	mean	±SD	SpitWorm	MecWorm	SpitWorm	SpitWorm	MecWorm
1	α-Pinene	938	937	0.36	0.23	0.23	0.20	0.10	0.15	0.92	0.72	0.0808242	0.4924009	0.4063556	n.s.	n.s.	n.s.	
2	Octen-3-ol	980	980	9.55	4.16	33.80	8.94	10.18	5.75	0.46	0.46	0.9849010	0.0000076	0.0000106	n.s.	***	***	
3	3-Octanone	988	986	0.35	0.48	4.64	1.69	1.98	2.87	n.d.	-	0.3380395	0.0039585	0.0931260	n.s.	**	n.s.	
4	Myrcene	993	991	0.58	0.20	1.15	0.74	0.22	0.26	0.62	0.30	0.4323617	0.2146452	0.0166856	n.s.	n.s.	*	
5	3-Octanol	996	994	1.74	0.61	3.63	0.83	1.00	1.62	1.30	1.39	0.4994582	0.0341605	0.0026187	n.s.	*	**	
6	Decane	1000	1000	9.62	3.30	0.08	0.17	0.33	0.41	9.93	10.49	0.0000002	0.0000001	0.9643412	***	***	n.s.	
7	Octanal	1004	1003	0.60	0.63	0.95	1.22	0.63	0.96	0.42	0.47	0.9981364	0.8465540	0.8767897	n.s.	n.s.	n.s.	
8	(Z)-3-Hexenyl acetate	1007	1005	41.15	17.68	114.52	23.65	25.43	15.92	0.67	0.54	0.3497002	0.0000052	0.0000004	n.s.	***	***	
9	Hexyl acetate	1013	1011	1.30	0.65	7.25	2.63	0.95	0.62	0.05	0.14	0.9317235	0.0000098	0.0000048	n.s.	***	***	
10	(E)-2-Hexenyl acetate	1016	1016	3.27	1.60	20.70	7.26	3.38	2.50	0.02	0.03	0.9990825	0.0000054	0.0000058	n.s.	***	***	
11	o-Cymene	1028	1022	0.02	0.02	0.57	1.00	0.00	0.00	0.00	0.00	0.9979047	0.3277438	0.2981185	n.s.	n.s.	n.s.	
12	Limonene	1033	1030	9.90	3.54	0.18	0.15	0.27	0.37	11.27	10.02	0.0000004	0.0000001	0.9964716	***	***	n.s.	
13	(Z)-β-Ocimene	1039	1038	1.22	0.51	5.56	3.40	1.27	1.97	n.d.	-	0.9992930	0.0192015	0.0207784	n.s.	*	*	
14	(E)-β-Ocimene	1050	1049	29.85	11.62	132.88	71.46	37.59	48.59	0.03	0.08	0.9648645	0.0102197	0.0180574	n.s.	*	*	
15	Octanol	1071	1071	1.69	1.39	14.08	7.49	3.90	2.77	0.57	0.80	0.7254716	0.0012417	0.0071688	n.s.	**	*	
16	Linalool	1101	1099	7.24	4.27	15.24	4.72	3.66	2.77	0.66	0.79	0.2893966	0.0061191	0.0001822	n.s.	*	***	
17	Nonanal [§]	1105	1104	2.98	1.42	5.06	3.11	4.11	2.55	1.85	0.82	0.7148857	0.3792424	0.8605077	n.s.	n.s.	n.s.	
18	DMNT	1118	1116	5.25	2.75	52.16	32.35	16.36	23.10	0.00	0.00	0.7276463	0.0186261	0.0936601	n.s.	*	n.s.	
19	Myroxide	1144	1141	3.52	2.89	5.76	1.84	1.82	0.83	n.d.	-	0.3382861	0.2031474	0.0102794	n.s.	n.s.	*	
20	(Z)-3-Hexenyl butanoate	1187	1185	3.26	1.23	7.57	2.91	1.86	1.15	0.05	0.14	0.4565772	0.0034609	0.0002205	n.s.	**	***	
21	Methyl salicylate	1202	1192	0.27	0.65	5.99	7.46	0.78	1.01	0.00	0.00	0.9800192	0.1411725	0.1991379	n.s.	n.s.	n.s.	
22	Decanal	1207	1206	8.59	5.18	10.41	6.86	14.11	13.98	4.94	1.75	0.5548678	0.9514157	0.6932095	n.s.	n.s.	n.s.	
23	2-Phenoxyethanol	1225	1226	1.42	0.65	4.14	2.10	3.16	1.05	1.39	0.88	0.1671388	0.0349239	0.7620247	n.s.	*	n.s.	
24	(Z)-3-Hexenyl-alpha-methylbutyrate	1234	1234	2.38	1.16	7.43	4.11	3.05	5.04	0.04	0.08	0.9473481	0.0634960	0.1181969	n.s.	n.s.	n.s.	
25	(Z)-3-Hexenyl isovalerate	1237	1238	0.87	0.89	6.97	3.38	3.22	2.89	0.00	0.00	0.3756139	0.0111147	0.1889186	n.s.	*	n.s.	
26	Nonanoic acid [§]	1264	1267	0.03	0.05	0.88	0.41	0.08	0.14	0.18	0.23	0.9324190	0.0000791	0.0001713	n.s.	***	***	
27	Indole	1301	1295	3.75	1.74	20.30	9.26	10.90	7.64	0.57	0.72	0.2362976	0.0032837	0.1267038	n.s.	**	n.s.	
28	(E)-3-Hexenyl tiglate [§]	1326	1315	1.08	0.29	4.56	3.10	2.65	2.64	0.55	0.38	0.5034148	0.0253788	0.2392582	n.s.	*	n.s.	
29	(E)-2-Hexenyl tiglate	1339	1339	0.06	0.13	0.99	0.90	0.24	0.26	0.07	0.10	0.8509421	0.0117840	0.0388339	n.s.	*	*	
30	α-Ylangene [§]	1389	1373	0.28	0.24	0.06	0.07	0.33	0.75	0.00	0.01	0.9813802	0.6737437	0.5540261	n.s.	n.s.	n.s.	
31	Tetradecane	1400	1400	0.19	0.06	0.45	0.08	0.96	0.38	0.26	0.10	0.0000272	0.1278173	0.0007499	***	n.s.	**	
32	Jasmone	1408	1394	1.10	0.72	2.89	1.65	2.23	3.47	0.06	0.13	0.6421530	0.2575832	0.7860770	n.s.	n.s.	n.s.	
33	(E)-β-Caryophyllene	1437	1420	0.23	0.21	1.73	1.29	0.26	0.38	n.d.	-	0.9968881	0.0076340	0.0090474	n.s.	*	*	
34	Geranyl acetone	1457	1453	2.32	1.68	4.35	3.60	5.87	4.01	1.16	0.74	0.1680678	0.5979240	0.5547825	n.s.	n.s.	n.s.	
35	β-Ionone	1496	1491	0.02	0.03	1.41	0.30	0.15	0.20	0.00	0.00	0.5898963	0.0000000	0.0000000	n.s.	***	***	
36	Pentadecane	1500	1500	0.82	0.19	0.98	0.63	27.79	13.76	0.80	0.49	0.0000250	0.9999390	0.0000112	***	n.s.	***	
37	δ-Jasmolactone	1503	1518	0.25	0.22	2.61	1.85	2.93	5.66	0.18	0.31	0.3561603	0.3337577	0.9985786	n.s.	n.s.	n.s.	
38	TMTT	1583	1577	0.36	0.37	2.69	1.95	1.45	1.31	0.00	0.01	0.4170494	0.0471015	0.4626453	n.s.	*	n.s.	
		N		6		7		6		8		Different			4		23	
												Not Different			34		15	
												of total			38		38	

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202 A principal component analysis (PCA) of all treatments and relative amounts of all 38 compounds
 203 revealed significant differences between MecWorm treatment on one hand and SpitWorm and *S.*
 204 *littoralis* larval treatment on the other hand, whereas confidence areas (95%) of SpitWorm and *S.*
 205 *littoralis* treatments overlap almost completely (Figure 7). The two principal components PC1 and PC2
 206 explain 55.2% of all observed variances. One-way ANOVA followed by Tukey's HSD as post hoc test
 207 for each compound comparing all treatments showed that between larval and SpitWorm treatment the
 208 relative amounts of only four compounds out of 38 were significantly different. In contrast the mean
 209 values of 23 compounds differ significantly between larval and MecWorm treatment (Table 1, S5 Fig).

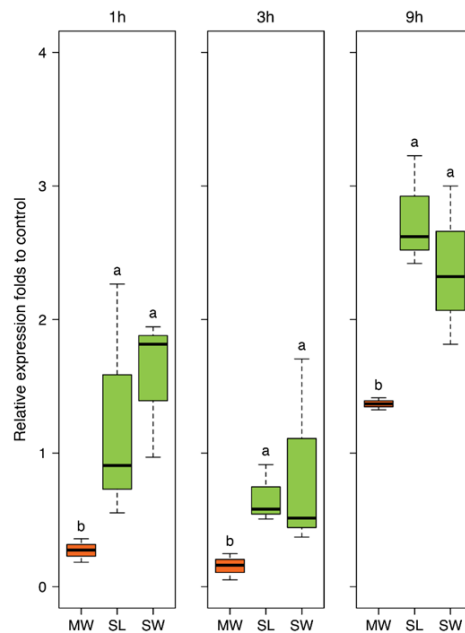


210

211 **Figure 7.** Principal component analysis of relative amounts of 38 volatiles released by different
 212 treatments. Three different treatments on lima bean leaves (*S. littoralis* larva, n = 6; MecWorm, n = 7;
 213 SpitWorm, n = 6). PC, principal component (% of total variance); confidence area, 95%.

214 2.9. Comparative Quantitative real-time RT-qPCR

215 For all time periods the four different JA responsive genes tested (lipoxygenase (*LOX3*),
 216 phenylalanine ammoniolyase (*PAL*), β -1, 3-glucanase (*PR2*), chitinase (*PR3*)) showed no significant
 217 differences in expression levels between *S. littoralis* and SpitWorm treatment. In cases where larval
 218 treatment resulted in a significant difference compared to sole mechanical wounding by MecWorm,
 219 SpitWorm and larval treatment showed a stronger induction (Figure 8 and Figure S6).



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Figure 8. Expression of *PAL* in lima bean leaves after different treatments. Lima beans treated for 1 h, 3 h and 9 h with MecWorm (MW), *S. littoralis* (SL), and SpitWorm (SW; 10 times diluted OS, delivery speed of 10 nL·s⁻¹); n = 3 for each treatment, log₂ transformed, one-way ANOVA, post-hoc test: Fisher's LSD, treatments with identical letters are not significantly different.

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3. Discussion

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Plants react to herbivory with a series of defense reactions provoked by the mechanical destruction of plant tissue in combination with chemical compounds left by the feeding organism. For lima bean leaves, it has been shown that sole continuous mechanical wounding by a designed mechanical caterpillar (MecWorm) is able to induce volatile emissions qualitatively almost identical to the bouquet released upon herbivory by *S. littoralis* larvae. This induction was not observed by wounding with razor blades or pattern wheels [8,20].

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In order to be able to study the influence of larval oral secretions or single chemical compounds on the leaf's wounding response, we aimed for turning the mechanical caterpillar MecWorm into SpitWorm, which combines the mimicking of mechanical leaf wounding by a larva with a continuous and simultaneous delivery of larval OS through a capillary to the tip of the punching needle. The feeding of *S. littoralis* larvae on lima bean leaves and the respective defense response of the plant was chosen as the 'gold standard' model for developing and parameter-adjusting of SpitWorm.

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The average leaf wounding sizes of larval feeding were measured for different feeding periods (Figure 3a) and the continuous mechanical leaf damage by SpitWorm was set accordingly for experiments comparing larvae and SpitWorm treatment. For SpitWorm fluid delivery an optimized flow rate of 10 nL·sec⁻¹ was evaluated to leave a continuous trail during mechanical 'biting'. It is worth to note that the values for flow rate as well as for biting rate and the leaf area destroyed per bite of SpitWorm are different to real larval feeding.

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In order to determine the amount of OS left by a larva at the wounding edge and to adjust the amount of OS to be delivered by SpitWorm a fluorescent dye solution (Lucifer Yellow CH) was injected into the foregut of the larvae before they start feeding on leaves. Lucifer Yellow CH was chosen because (i) of its fluorescence emission maximum at 535 nm, fitting perfectly in the green gap between 490 to 620 nm of chlorophyll a and b, (ii) it is assumed to be non-toxic, (iii) of its high quantum yield, and (iv) it is highly dissociated at physiological pH levels [38]. The injected dye solution starts to move through the whole body of the larva to the anus about 45 min to 1 hour after injection (Figure 4). Therefore, all feeding experiments had to start within 30 mins after injection. After comparing the feeding behavior of larvae injected with different volumes of fluorescent dye solution with untreated larvae (Figure 3b), an injection volume of 5 µL was chosen for subsequent experiments because higher injection volumes

254 resulted in a significant reduction of larval feeding activities. A long-time effect of Lucifer Yellow on
255 further larval growth and development was not evaluated in this study because all experiments with
256 fluorescence labeled larvae were completed within less than 1 h after injection.

257 Comparison of microscope fluorescence images of the wounding edges of leaves treated with
258 labeled larvae, SpitWorm delivering fluorescent labeled OS (dilution. 1:10; delivery rate, 10 nL·s⁻¹), and
259 on the other hand treated with MecWorm and with a razor blade, both without any fluorescent labeling
260 showed (i) a pronounced fluorescence signal at the wounding edge after larva and SpitWorm treatment
261 (Figures. 5A-B) and (ii) no fluorescence after sole mechanical wounding by MecWorm or a razor blade
262 (Figures. 5C-D). This confirms that larval OS infiltrates the tissue at the wounding edge and that the
263 fluorescence signal is not resulting from or affected by sole mechanical wounding. SpitWorm left a
264 slightly different pattern of OS trail at the edge of the wounding site compared to larva feeding. Upon
265 larval feeding, the OS goes deeper into the plant tissue following the veins compared to SpitWorm
266 treatment. The wounding edges showed a difference in biting patterns. In accordance with scanning
267 electron micrographs of wounding sites resulting from MecWorm and larva treatment reported earlier
268 [20], larva feeding forms a straight borderline in contrast to SpitWorm treatment, which forms to some
269 extend a small, frayed zone which can explain the different permeation depths of the fluids.

270 The amount of OS left by a larva per bite (approximately 50 to 100 pL·bite⁻¹) was calculated by
271 quantifying the fluorescent dye extracted from the wounding edge tissues of leaves treated with labeled
272 larvae in combination with feeding duration, average foregut volume, amount of fluorescent dye
273 solution injected, and observed larval biting rates (3-5 bites·s⁻¹). This resulted in a calculated flow rate
274 of 250 - 300 pL·s⁻¹ for the OS delivered by a larva which is 30 – 40 times lower than the optimized flow
275 rate (10 nL·s⁻¹) determined for SpitWorm (see above). Thus, the 'effective' OS delivered by SpitWorm
276 had to be aligned to the amount of OS from the larva during feeding by dilution. The amount of
277 fluorescent dye extracted from larva-damaged tissue (see above) was compared with the amounts
278 extracted from wounding edge tissues after SpitWorm treatment delivering different dilutions of
279 labeled larval OS labeled with Lucifer Yellow solution (Figure 6). This resulted in an optimized dilution
280 of 1:10 of larval regurgitate for SpitWorm treatments. The concentration of fluorescent dye in the
281 regurgitate before dilution was adjusted to the concentration in the larval foregut.

282 With an OS dilution of 1:10 SpitWorm delivers an equivalent of 1 nL OS per second, which is still
283 about three to four times the amount of OS left by larva feeding (0.3 nL·s⁻¹). Here it needs to be
284 considered that the feeding track of *S. littoralis* larvae is not linear but usually follows repetitively a
285 curved path. The OS left in the tissue during a feeding bout is re-ingested by the larva in the next round
286 [39], while SpitWorm is continuously delivering diluted OS without taking up the damaged plant
287 tissue. Additionally, to reduce the viscosity caused by large polysaccharides, proteins, fat and food
288 residues, freshly harvested regurgitate was filtered through a 0.22 µm filter and it cannot be excluded
289 that active components were removed thereby.

290 Delivery of OS by SpitWorm was conducted at room temperature. Although the larvae were raised
291 healthily within the same temperature range, it is not ensured that all the active compounds in the OS
292 can keep the same activity, especially with long time delivery. This problem may be compensated by
293 an over-delivery of OS; nevertheless, more experiments need to be done in the future to test the
294 activities of chemical factors by using SpitWorm.

295 In order to test to what extend SpitWorm can mimic larva feeding, with all the parameters
296 evaluated so far, relative VOC amounts in the headspace of leaves as well as expression levels of four
297 JA responsive genes in leaves upon larvae, MecWorm, and SpitWorm treatment were compared.

298 Volatiles in the headspace of lima bean leaves released upon different treatments were collected,
299 identified, and quantified relative to an internal standard by GC-MS (Table 1). Instead of charcoal as
300 described earlier for comparison of MecWorm and larvae treatment [8,20] we used Porapak Q as
301 adsorbent for a more reliable quantification. It is known that for example (*E*)-β-ocimene, one of the
302 most abundant compounds induced after larvae treatment of lima bean leaves, is oxidized to (3*E*,5*E*)-
303 2,6-dimethyl-3,5,7-octatrien-2-ol and dehydrogenated to (3*E*,5*E*)-2,6-dimethyl-1,3,5,7-octatetraene to
304 some extend when collected on active charcoal in the presence of humid air [40]. Besides these two a
305 third artefact, 5-ethylfuran-2(5H)-one (described as hexenolide by Bricchi *et al.* [8]), only detected after

306 continuous mechanical wounding by MecWorm but not upon larval treatment, was absent when using
307 Porapak Q as adsorbent. In total 38 compounds in all three treatments were identified and their relative
308 amounts were subjected to a principal component analysis which revealed an almost complete overlap
309 of the confidence areas (confidence level, 95%) resulting from larvae and SpitWorm treatment. The
310 cluster of the MecWorm treatments is clearly separated (Figure 7), showing that SpitWorm mimics
311 larval feeding much better than MecWorm. Comparing the relative amounts of each compound after
312 SpitWorm and larvae treatment shows that 90% are not significantly different. On the other hand,
313 comparing MecWorm with larval treatment, the relative amounts for 60% of the compounds exhibit a
314 significant difference (Table 1). In general, MecWorm treatment evoked a stronger plant response, i.e.
315 higher amounts of compounds released, than larvae or SpitWorm treatment (Figure S5). This leads to
316 the conclusion, that in this case, mechanical wounding is responsible for inducing volatile emission
317 upon herbivory, figuratively named ‘the cry for help’, but compounds in the OS reduce the emission to
318 ‘turn down the sound’.

319 To further investigate responses to SpitWorm treatment in *P. lunatus* leaves, time series of
320 expression levels for four JA-responsive genes were chosen which were also used in earlier studies [41].
321 The four genes are encoding for: lipoxygenase (*LOX3*), phenylalanine ammonia-lyase (*PAL*), and the
322 pathogenesis-related (PR) proteins (*PR2* (β -1, 3-glucanase)) and (*PR3* (chitinase)).

323 All treatments showed no significant difference between SpitWorm treatment and larvae feeding
324 (Figure S6). Whereas for *PAL* SpitWorm/*S. littoralis* treatments resulted in a stronger induction for all
325 periods compared to sole continuous mechanical wounding by MecWorm (Figure 8) the expression
326 levels of the other JA responsive genes showed no significant differences between the different
327 treatments for longer periods. Except for *LOX* where almost no difference between mechanical
328 wounding and larval feeding is observed, the early response (1 h) is influenced by OS from *S. littoralis*
329 or SpitWorm, respectively. This indicates that mechanical wounding alone is able to induce the JA
330 responsive genes pathway, but chemical factors enhance or modulate this induction for a more rapid
331 defense response. Additionally, the results confirmed that 10 times diluted OS is the optimal dilution
332 factor to add OS to SpitWorm to mimic *S. littoralis* feeding.

333 As a further developed MecWorm, a SpitWorm was expected to mimic the action of a feeding
334 insect as close as possible. The results emphasize that both, mechanical wounding and chemical factors
335 play prominent roles in gene regulation and defense reactions, which further proves that SpitWorm
336 can be used as an effective tool in mimicking insect feeding. Our findings also support the hypothesis
337 that in wounded leaves, mechanical wounding can trigger most of the defense reactions while chemical
338 factors in insect OS have a ‘fine-tune’ function by enhancing or attenuating the induction of gene
339 expression by mechanical wounding.

340 With this new tool at hand, it is now possible to study the interplay of mechanical wounding and
341 larval OS at different environmental conditions or with different combinations of compounds. Using
342 fractions of larval OS or single compounds will allow tracking down individual elicitors and in
343 combination with other comparative genomic, transcriptomic or proteomic methods, it will be possible
344 to go further and deeper in understanding regulation processes of plant defense against herbivory.

345 4. Materials and Methods

346 4.1. Plant and insect materials

347 Plants

348 Lima bean, *Phaseolus lunatus* L. (Ferry Morse cv. Jackson Wonder Bush) was grown from seeds at
349 23 °C and 60% humidity in plastic pots (diameter 5.5 cm) using sterilized potting soil. For daylight
350 radiation, fluorescent tubes (ca. 270 $\mu\text{E m}^{-2}\text{s}^{-1}$) with a photophase of 16 h were used. Experiments were
351 conducted with 12 to 16 days old seedlings showing two fully developed primary leaves.

352 Insects

353 *Spodoptera littoralis* (Lepidoptera, Noctuidae) larvae, hatched from eggs (Bayer CropScience AG,
354 Monheim, Germany) were reared on artificial diet (500 g white beans powder soaked overnight in 1.2

355 L water, 9 g ascorbic acid, 9 g parabene, 4 mL formaldehyde (36.5%), and 75 g agar boiled in 1.0 L of
356 H₂O) and raised at 22 °C to 24 °C, 14 h to 16 h photophase, to the developmental stage of 3rd to 5th instar.
357 For all experiments, larvae with a body length in the range of 2.5 – 3 cm were chosen.

358 4.2. SpitWorm

359 A gas-tight glass syringe (100 ml) was connected to a capillary (Fused Silica, 0.25 mm i.d., SGE,
360 Melbourne, Australia) running from the top of the punching head of MecWorm [20] through the inner-
361 hollow of the 'biting' needle until to a hole close to the needle tip. The syringe was actuated by a syringe
362 pump (Harvard Apparatus PHD 2000) to generate a stable and quantitative fluid delivery (Figure 1).

363 4.3. Flow rate optimization

364 In order to determine the lowest flow rate at which a fluid can be supplied continuously without
365 interruption through the hole in the punching needle, ink was used instead of larval OS. With different
366 delivery rates (2.5 nL·s⁻¹, 5 nL·s⁻¹, 10 nL·s⁻¹) SpitWorm was set to mimic larval biting pattern on a filter
367 paper for 5 min.

368 4.4. Collection of insect oral secretions

369 Regurgitate was collected by slight squeezing the larvae with tweezers and collection of the
370 excreted fluid with a Gilson Pipetman P20 Variable Volume Pipette (2 to 20 µL). If not used
371 immediately, the regurgitate was frozen and stored at -20 °C. Before dilution and use with SpitWorm,
372 larval regurgitate was filtered through a syringe filter (CME, 0.22 µm).

373 4.5. Wounding size and biting rate determination

374 After 12 h of starving single larvae were allowed to feed on a lima bean leaf for 5 min, 1 h, 3 h, 9 h
375 and 17 h. For subsequent quantification of wounding areas, only treatments where the larva did not
376 feed at the leaf edges were used. Images of the damaged leaves were printed out. Scaled unit areas and
377 wounding areas were cut out of the same sheet of paper and weighed. Wounding sizes were
378 determined by dividing the paperweight of the wounding area by the paperweight of the scaled unit
379 ($n = 4$ for each treatment).

380 Biting rate (bites·s⁻¹) was determined by evaluating a close-up slow-motion video of a larva during
381 feeding on a lima bean leaf.

382 4.6. Insect foregut volume determination

383 After feeding, larvae were suffocated in 75% ethanol solution for 30 seconds, dissected, and the
384 length (l) and width (d) of the foregut were measured. Foregut volume (V_g) was calculated as a cylinder
385 ($V_g = l \cdot \pi \cdot (d/2)^2$, $n = 5$).

386 4.7. Optimization of the injection volume of fluorescent dye solution

387 After being starved for 12 hours, larvae were injected with 1 µL, 5 µL, 10 µL, and 15 µL of an
388 aqueous solution (1 mg·mL⁻¹) of Lucifer Yellow CH dipotassium salt (Fluka, $\lambda_{Ex} = 428$ nm, $\lambda_{Em} = 535$
389 nm), 4 replicates for each injection volume. As control, 4 starved larvae without being injected were
390 used. Larvae were then fed on lima bean plants for 5 min, wounding areas were pictured with a LEICA
391 LMD6000 fluorescence microscope and wounding sizes were measured as described above.

392 4.8. Fate of foregut injected fluorescent dye

393 In order to estimate the residence time of the injected fluorescent dye solution in the larval foregut,
394 *S. littoralis* were injected with 5 µL of an aqueous solution (1 mg·mL⁻¹) of Lucifer Yellow and observed
395 under ultraviolet light (365 nm) for 3 h.

396 4.9. Amount of OS left on the leaf at the wounding zone

397 An aqueous solution (5 μL , 1 $\text{mg}\cdot\text{mL}^{-1}$) of Lucifer Yellow CH dipotassium salt was injected into
398 the larval foregut. After injection, larvae were allowed to feed on leaves for 5 min. Leaf tissue around
399 the wound margins was cut out, ground in liquid nitrogen, suspended in 1 mL H_2O by shaking for 1 h
400 in the dark at 4 $^{\circ}\text{C}$, and centrifuged for 10 min at 12.6×1000 rcf. The supernatant was subjected to
401 fluorescence signal quantification with a FP-750 Spectrofluorometer (JASCO Cooperation, Tokyo,
402 Japan). The supernatant of centrifuged tissue suspensions of leaves treated with unlabeled larvae,
403 processed in the same manner as above served as blank and as solvent for the dilutions for the standard
404 curve measurements ($n = 3$ for each treatment). A standard curve ($R^2 = 0.9968$; Figure S4) was generated
405 using a series of dilutions (0, 5, 10, 15, 20, 25, and 30 $\text{nL}\cdot\text{mL}^{-1}$) of Lucifer Yellow solution (1 $\text{mg}\cdot\text{mL}^{-1}$).

406 4.10. Optimization of OS dilution for SpitWorm treatment

407 Leaves were treated for 5 min with SpitWorm (wounding size, 0.30 cm^2 ; fluid delivery rate, 10
408 $\text{nL}\cdot\text{sec}^{-1}$) using different aqueous dilutions (1:5, 1:10, 1:30) of fluorescence dye labeled regurgitate
409 (Lucifer Yellow solution (5 μL , 1 $\text{mg}\cdot\text{mL}^{-1}$) in 44 μL of filtered larval regurgitate).

410 For fluorescence signal quantification ($n = 3$ for each treatment), tissues of the wound edges were
411 processed in the same manner as described for the treatment with labeled larvae (see above).

412 4.11. Fluorescence microscope imaging

413 Lima bean leaves were treated by *S. littoralis* larva (injected with Lucifer Yellow solution, 5 μL , 1
414 $\text{mg}\cdot\text{mL}^{-1}$), SpitWorm (delivering a diluted (1:10) Lucifer Yellow solution (5 μL , 1 $\text{mg}\cdot\text{mL}^{-1}$) in 44 μL of
415 filtered larval regurgitate at a flow rate of 10 $\text{nL}\cdot\text{s}^{-1}$) and by MecWorm and a razor blade, respectively,
416 both without fluorescent labeling. Pictures of the wounding areas were taken with a LEICA LMD6000
417 fluorescence microscope.

418 4.12. Collection and analysis of headspace volatiles

419 For headspace volatiles collection of *S. littoralis* treated (one larva per leaf) as well as for MecWorm
420 and SpitWorm (filtered larval OS, 1:10 diluted; flow rate, 10 $\text{nL}\cdot\text{sec}^{-1}$) treated leaves (equivalent duration
421 (17 h) and wounding areas (7.25 cm^2) for all treatments), the test leaves were enclosed in an acryl glass
422 case (width \times depth \times height, 95 \times 87 \times 135 mm^3 ; net headspace volume approximately 1 L) together
423 with the punch head in the MecWorm device (Figure 2). The capillary of SpitWorm was threaded
424 through a hole (diameter, 0.5 mm) in the left sidewall of the case; the stainless steel tubings of the
425 volatile collection pump system were inserted through two holes (diameter, 0.8 mm) in the top-side.
426 For control (untreated leaves) and larvae treatment, the punching head was positioned away from the
427 leaf area and the device was switched off. The wounding time and area were 17 h and 7.25 cm^2 ,
428 respectively. Headspace volatiles emitted by untreated lima bean leaves ($n = 8$), or treated with larvae
429 ($n = 6$), MecWorm ($n = 5$), and SpitWorm ($n = 6$), respectively, were continuously collected for 24 h on
430 Porapak Q traps (quartz glass tubes ; length, 66 mm; inner diameter, 2.5 mm; outer diameter, 5.5 mm;
431 filled with 10 mg Porapak Q, 80-100 mesh, Aldrich) using closed-loop-stripping (CLS) method [42].
432 Traps were pre-cleaned before the first use and regenerated after elution by rinsing with 1 ml of solvent
433 each in the following order: methanol, methanol/chloroform (1:3), chloroform, acetone,
434 dichloromethane and dried at 60 $^{\circ}\text{C}$ for 24 h. All experiments were started between 11:00 and 13:00.
435 Setups were kept at 22 - 24 $^{\circ}\text{C}$ with a light/dark rhythm of 7 h light, 10 h dark, and 7 h light. For all
436 samples after volatile collection, adsorbed compounds were eluted with dichloromethane ($2 \times 50 \mu\text{L}$,
437 supplemented with 1-bromodecan (50 $\mu\text{g}\cdot\text{ml}^{-1}$) as internal standard, and stored at -20 $^{\circ}\text{C}$ prior to
438 analysis. Samples were analyzed by gas chromatography mass spectrometry (GC-MS) with an ISQ GC-
439 quadrupole MS system (Thermo Scientific, Bremen, Germany) equipped with a fused silica capillary
440 column ZB-5 (30 m \times 0.25 mm \times 0.25 μm with 10 m guard column, Zebron, Phenomenex, USA). Helium
441 at 1 $\text{mL}\cdot\text{min}^{-1}$ served as carrier gas with an injector temperature of 220 $^{\circ}\text{C}$ running in split mode (1:10);
442 1 μL of sample was injected. Separation of the compounds was achieved under programmed
443 temperature conditions (45 $^{\circ}\text{C}$ for 2 min, then at 10 $^{\circ}\text{C}\cdot\text{min}^{-1}$ to 200 $^{\circ}\text{C}$, then at 30 $^{\circ}\text{C}\cdot\text{min}^{-1}$ to 280 $^{\circ}\text{C}$ kept
444 for 1 min). The MS was run in EI mode (70 eV) with a scan range of 35 to 450 amu, a transfer line

445 temperature of 280 °C, and an ion source temperature of 250 °C. Data acquisition was performed using
446 Xcalibur 3.1 (Thermo Fisher Scientific).

447 A mixture of n-alkanes C₈ – C₂₀ in *n*-hexane (Aldrich) was measured before and after a sample
448 sequence under the same conditions except for the injector split ratio (1:50). Retention times of the *n*-
449 alkanes were used to calculate the retention index (RI) for each peak in the GC-MS chromatogram
450 according to the method of [van Den Dool, et al. \[43\]](#).

451 Compounds were identified based on their mass spectra (MS) in combination with their individual
452 RIs in comparison to [NIST \[36\]](#), [Adams \[37\]](#) and [Massfinder \[44\]](#) MS and RI databases using Massfinder
453 software in combination with NIST MS Search. Authentic reference compounds were used additionally
454 for identification, if at hand. For relative quantification, identified peaks of the GC-MS total ion
455 chromatogram (TIC) were integrated and the peak areas were divided by the peak area of the internal
456 standard.

457 4.13. One-Step comparative RT-qPCR

458 For gene expression analysis, lima bean leaves were treated by larvae, MecWorm, and SpitWorm
459 (flow rate, 10 nL·s⁻¹; OS dilution, 1:10) for 1, 3, and 9 hours with comparable leaf wounding sizes for
460 each period. Leaves of untreated plants served as control. Three technical and three biological replicates
461 were used for each sample.

462 Primers for RT-qPCR were designed with Primer3plus [\[45\]](#) according to gene sequences from
463 *Phaseolus vulgaris*: Lipoxygenase (*LOX3*, X63521, [\[46\]](#)) in the octadecanoid pathway; phenylalanine
464 ammonialyase (*PAL*, M11939, [\[47\]](#)) in the phenylpropanoid pathway; and pathogen-related (PR)
465 proteins (*PR2* (β-1,3-glucanase), X53129, [\[48\]](#)) and (*PR3* (chitinase), M13968, [\[41,48,49\]](#)). *P. lunatus* actin
466 (*PACT1*, DQ159907) gene was used as the normalizer [\[50\]](#). OligoAnalyzer 3.1 was used for primer
467 analysis.

468 Primers used for RT-qPCR were as follows were follows:

469	<i>LOX3</i> (X63521),	forward	5'-TGGATGACCGATGAAGAA-3',
470		reverse	5'-TGTTGCTATGACGAATGG-3';
471	<i>PAL</i> (M11939),	forward	5'-GAAACCTTAGAATCCATCACCA-3',
472		reverse	5'-TAGAAGCCAAGCCAGAACC-3';
473	<i>PR2</i> (X53129),	forward	5'-AAACTCCTACCCTCCATCACAA-3',
474		reverse	5'-CCATCCCTCACCACAACA-3';
475	<i>PR3</i> (M13968),	forward	5'-GGCGAGGACAGGATAGCAG-3',
476		reverse	5'-TCACAAAGGGAAACACAGATT-3'
477	<i>PACT1</i> (DQ159907),	forward	5'-AGGCTCCTCTTAACCCCAAG-3',
478		reverse	5'-GTGGGAGAGCATAACCCTCA-3'.

479 Leaf tissues (80 - 100 mg) were collected around the leaf's wounding edges or from intact leaves
480 as control and ground in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen)
481 following the manufacturer's protocol and purified using TURBO™ DNase (Ambion) and RNeasy
482 MinElute Cleanup kit. RNA was then directly subjected to 1-step Comparative quantitative real-time
483 RT-qPCR using the Verso™ SYBR Green 1-Step QRT-PCR Low ROX Kit (ABgene), with an Mx3000P
484 Real-Time PCR system (Stratagene). The process was conducted according to the manufacturer's
485 protocol with a 25 µL reaction system, consisting of 0.25 µL Verso Enzyme Mix, 12.5 µL 1-Step qPCR
486 SYBR Mix, 1.25 µL RT Enhancer, 1.75 µL forward and reverse primers (1 µM) each, 2 µL RNA template
487 (25 ng·µL⁻¹) and 5.5 µL water (PCR grade). The reaction procedure was as follows: cDNA synthesis for
488 15 min at 50 °C, Thermo-Start activation for 15 min at 95 °C followed by 40 cycles of denaturation (15 s
489 at 95 °C), annealing (30 s at 55 °C for *PR2* and *PAL*; 30 s at 60 °C for *LOX3* and *PR3*), and extension (30
490 s at 72 °C). Fluorescence signals were recorded after each annealing step. After this an additional
491 temperature cycle (95 °C, 30 s and 60 °C, 30 s) was followed by an incremental heating to 95 °C (stepwise
492 0.5 °C for 10 s) to verify the products by a dissociation curve. Fluorescence signals were recorded during
493 the whole melting process. PCR conditions were determined by comparing threshold values, followed
494 by non-template control for each primer pair. Relative RNA levels were normalized with the level of *P.*

495 *lunatus* actin mRNA (*PACT1*) and calibrated with relative expression levels of the target genes in
496 untreated control plants.

497 4.14. Statistical analysis

498 Statistical analysis was performed using the free software package R version 3.4.3 [51] in
499 combination with RStudio version 1.1.423 [52]. The level of statistical significances among means of
500 different group values was evaluated by one-way analysis of variance (one-way ANOVA) followed by
501 post-hoc tests (Tukey's HSD test, Fisher's LSD) for multiple comparisons. RT-qPCR data were log₂
502 transformed and Fisher's LSD was used as post-hoc test.

503 For comparing the relative amounts of volatile compounds released upon different treatments, a
504 dimension reduction by principal component analysis (PCA) with scaled experimental values was
505 performed.

506 **Supplementary Materials:** The following are available online at <http://www.mdpi.com/xxx-xxx>, Figure S1:
507 Workflow for determination of OS amount left at the leaf wounding edges, Figure S2: Comparison of fluorescence
508 signals left in plant wounded sites by insects injected with fluorescent dye, Figure S3: SpitWorm set-up and flow
509 rate optimization, Figure S4: Standard curve of fluorescence signal intensity of different dilutions of Lucifer Yellow
510 solution (1 mg·mL⁻¹), Figure S5: Comparison of relative amounts of headspace volatiles upon different treatments,
511 Figure S6: Expression of four JA responsive genes (*LOX3*, *PAL*, *PR2*, and *PR3*), Table S1: Dimensions of larval
512 foreguts.

513 **Author Contributions:** GL, AM and WB designed the research, GL developed and optimized all methods and
514 performed all measurements and analyses. Volatile collection, GC-MS measurement and analysis was done by GL,
515 HG, MK, and SB. Statistical analysis and original draft preparation was conducted by GL and SB. SB, AM, WB
516 were involved in reviewing and editing the final manuscript.

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