Priming of cowpea volatile emissions with defense inducers enhances the plant's attractiveness to parasitoids when attacked by caterpillars

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Running title: Defense inducers increase the attractiveness of caterpillar-damaged cowpea to parasitoids

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ISS and TCJT conceived the ideas and designed the experiments. ISS performed the experiments and collected the data. ISS, TJAB and TCJT analyzed and interpreted the data. ISS, TJAB and TCJT wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication. The authors have declared that no competing interests exist.

ABSTRACT

BACKGROUND: The manipulation of herbivore-induced volatile organic compounds (HI-VOCs) *via* the application of the inducers BTH [benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester] and Laminarin [β-1,3-glucan] is known to enhance the attractiveness of caterpillar-damaged cotton and maize plants to parasitoids. To test if this is also the case for legumes, we treated cowpea with these inducers and studied the effects on HI-VOC emissions and the attraction of three generalist endoparasitoids.

RESULTS: After applying the inducers and subjecting the plants to either real or mimicked herbivory by *Spodoptera littoralis* caterpillars, females of the parasitoids *Campoletis sonorensis* and *Microplitis rufiventris* showed a strong preference for BTH treated plants, whereas *Cotesia marginiventris* females were strongly attracted to both BTH and Laminarin treated plants with real or mimicked herbivory. Treated plants emitted more of certain HI-VOCs, but considerably less indole, linalool and several sesquiterpenes. Multivariate data analysis revealed that enhanced wasp attraction after treatment was correlated with high relative concentrations of nonanal, α-pinene, (*E*)-β-ocimene and DMNT, and with low relative concentrations of indole, (*S*)-linalool and (*E*)-β-farnesene. Inducer treatments had no significant effect on leaf consumption by the caterpillars.

CONCLUSION: Our findings confirm that treating cowpea plants with inducers can enhance their attractiveness to biological control agents.

Keywords: Legumes; Indirect plant defense; Priming; Tritrophic interactions; Herbivory; Terpenoids; Indole.

1. INTRODUCTION

Plants have developed an effective arsenal of defense mechanisms during more than 400 million years of coevolution with insect herbivores and pathogens [1]. One of these defenses is the synthesis and release of a complex mixture of herbivore-induced volatile organic compounds (HI-VOCs) in response to herbivore attack [2]. These volatiles can mediate indirect defense by attracting the natural enemies of the herbivore [3,4].

In nature, plants are confronted with multiple attack scenarios involving pathogens and herbivores. Inducible defenses against these attackers are regulated by a complex network of phytohormonal signaling, in which the phytohormones salicylic acid (SA) and jasmonic acid (JA) are key players [5]. These hormones are also involved in the induction of plant volatiles [6], and crosstalk between SA and JA pathways will shape the eventual emission of induced VOCs [5,7], and thus the tritrophic interactions [8,9].

VOC induction can also be achieved by applying xenobiotic chemicals that act as inducers of plant resistance against herbivores or pathogens [10,11], causing qualitative or/and quantitative changes in the VOCs released [12]. An increasing body of evidence shows that the application of such plant defense inducers boosts indirect defense in various crop systems *i.e.*: cabbage [13]; cotton [12,14], lima bean [15]; maize [16–19]; sweet pepper [20]; rice [21,22]; soybean [23,24], and tomato [11,25]. In each case, treatment with an inducer alters the VOC blends, rendering plants more attractive to natural enemies of herbivores. Treating plants with these compounds can also enhance their direct defense against various herbivores [11]. Often such treatments do not immediately switch on defenses, but potentiates the plant so that it responds more quickly and more strongly when it is subsequently attacked by a herbivore[26]. This phenomenon, which is referred to as "priming" [26], is also known for HI-VOCs [27,28].

The development and application of inducers to promote resistance to herbivores is still at the experimental stage, but inducers that activate resistance against plant pathogens are already

being applied commercially [29,30]. Responses to inducer treatments vary strongly with plant genotype and rigorous experimental programs are needed to evaluate treatment effects in different crops before these agrochemicals can be used by growers for crop protection [31,32].

Legumes (Family: Fabaceae) are of particular interest in this context. They are second only to cereals in economic and nutritional value. Legumes provides on average 33% of human dietary protein, and as much as 60% in developing countries [33]. Cowpea, *Vigna unguiculata* L., is an important legume crop in semiarid areas because of its drought tolerance and adaptation to drier regions of the tropics, where other food legumes do not perform well [34]. Insect pests are still a major constraint to legume production in general [35], and can cause tremendous yield losses of up to 90% [36]. Integrated approaches to limit crops losses are being sought as alternatives to insecticides [37]. Biological control is an obvious alternative, but effectiveness needs to be improved so that it can be used on a larger scale [38]. The manipulation of VOC emission through the use of chemical inducers may help to improve the foraging efficiency of natural enemies and thus the biological control of insect pests [18,39].

The potential for this approach was shown for the plant strengtheners BTH (benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester) and Laminarin (β -1, 3 glucan). Both inducers are used to induce resistance to a wide range of diseases in field crops [29,30]. They induce the expression of pathogenesis related (PR) genes, but also enhance the attractiveness of plants to parasitoids [12,17]. This is only evident when primed plants are damaged; the treatment with BTH and Laminarin does not induce volatile emissions in intact plants [17]. Upon insect attack, primed plants, compared to unprimed plants, show increased emissions of some volatiles, but reduced emissions of other compounds that possibly interfere with or mask parasitoid attractants [16,17,40].

In the current study, we tested if the strategy can be extended to legume cropping systems by testing if the treatment with BTH and Laminarin of cowpea has similar beneficial effects as observed for maize and cotton [12,17]. For this, we conducted olfactometer assays with three generalist endoparasitoids: *Cotesia marginiventris* (Cresson), *Microplitis rufiventris* (Kok.) (Hymenoptera: Braconidae) and *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae), observing their

responses to the odors of cowpea plants primed with BTH and Laminarin. Plants were either infested with larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), as a real herbivory treatment, or mechanically damaged and treated with *S. littoralis* oral secretions (OS) as a mimicked herbivory treatment. *Spodoptera littoralis* is a generalist herbivore that causes severe damage to a wide range of fiber and forage crops in many agro-ecosystems [41]. To correlate parasitoid attraction with the emission of plant volatiles, the HI-VOC emissions of treated or untreated cowpea plants were also collected and analyzed. The leaf areas of cowpea plants consumed by *S. littoralis* caterpillars were measured to investigate the impact of inducer treatments on the direct defense of cowpea plants. With these experiments we addressed the following questions: (1) Does treatment of cowpea with BTH or Laminarin alter HI-VOC emission when plants are subsequently exposed to herbivores? (2) Do parasitoids respond differentially to HI-VOCs emitted from treated and untreated cowpea? (3) Does cowpea treatment with BTH or Laminarin affect the feeding behavior of *S. littoralis* caterpillars? The outcomes of the study could provide further insight into the potential of using chemical inducers as a sustainable and environmentally-benign strategy to enhance the biological control of insect pests in legume cropping systems.

2. MATERIAL AND METHODS

2.1 Plants and insects

Cowpea, *Vigna unguiculata* var. *unguiculata* (Haefliger AG®, Herzogenbuchsee, Switzerland) plants were grown in commercial potting soil (Ricoter, Aussaaterde, Aarberg, Switzerland) in plastic pots (11cm height, 4cm diameter) at 25± 2°C, 60 ± 5% r.h., 926 µmol/m^{2}s^1, 16:8h L:D, in a climate chamber (Percival, CLF plant climatics, Germany). Cowpea plants used for the experiments were 15 d-old and had 4-5 fully developed leaves. Eggs of *S. littoralis* were supplied by Syngenta® (Stein, Switzerland). Newly hatched larvae were reared in transparent plastic boxes on a wheat germ-based artificial diet until they had reached the second instar, at which point they were used for experiments. The larval

endoparasitoids, *M. rufiventris*, *C. marginiventris* and *C. sonorensis* were reared as described in Sobhy et al. [17].

2.2 Plant treatments

BTH (BION®) was obtained from Syngenta® (Basel, Switzerland) as a water-dispersible granular formulation containing 50% active ingredient. Laminarin (IODUS 40®) was obtained from Stähler (Zofingen, Switzerland) as a soluble liquid formulation containing 3.5 % active ingredient. Both BTH and Laminarin were sprayed with hand-held sprayer (McProper ®, 500 ml) on 13-day old plants 48 h before the experiment. The chemicals were applied at concentrations of 0.15 g/L (BTH) and 20 ml/L (Laminarin), which correspond to the doses recommended by the manufacturers for agricultural practices. Plants were sprayed with distilled water as a control treatment. For all treatments, each plant was sprayed to the point of run-off with ≈ 10 ml of inducer solution [42]. The different solutions were distributed equally over the whole plant. A day later, cowpea plants were challenged by either real or mimicked herbivory.

For the real herbivory treatment, plants were infested with ten 2nd instar larvae of *S. littoralis* placed on each plant 24 h after priming with elicitors. These plants were kept under laboratory conditions (25±2°C, 16:8 L/D) and were used in olfactometer assays the day after insects had been placed on the plants (Fig. 1). For the mimicked herbivory treatment, the abaxial side of two fully developed leaves (20 mm^2) was scratched using a scalpel blade, after priming with elicitors, and 10 µl of *S. littoralis* larval oral secretion (OS) was then applied to each wounded site using a micropipette (20 µl/plant). These secretions had previously been collected with a micropipette from 4th & 5th instar larvae that had been fed on cowpea leaves for at least 24 h, and were stored at -80°C until use [43]. This treatment was carried out twice: *i.e.* on the evening after inducer treatment and the next morning, one hour before starting the olfactometer experiment (Fig. 1).

2.3 Olfactometer bioassays

A series of experiments using a six-arm-olfactometer, as described by Sobhy et al. [12], was conducted to evaluate the effect of the treatments on the attractiveness of cowpea plants to parasitoids. More details of the six-arm-olfactometer such as the glassware dimensions, tubing and air delivery system are described in Turlings et al. [44]. For all experiments, 3-5 day old mated naïve female wasps were used. Thirty minutes before the bioassays, the parasitoid cage was transferred to the olfactometer laboratory for acclimatization. For the choice bioassays, a group of six female wasps was removed from their cage with an aspirator and released into the central choice chamber of the olfactometer. The wasps moved up to the top of the chamber attracted by the diffuse light coming from above. The airflow through each arm was approximately 400 ml/min (see below). The central choice chamber was connected *via* a Tygon tube to a water-filled glass U-tube that served as a pressure gauge to balance incoming and outgoing air; minimizing pressure differences with the outside [44].

Each group of wasps was given 30 min to make a choice. Wasps that did not enter an arm after this time were considered as individuals that made "no choice". After removing all wasps from the olfactometer a new group was introduced. Five groups of six wasps were tested on each experimental day, and each experiment was repeated 6 times on different experimental days, each time with a new set of treated plants as odor sources and with new parasitoids. At the end of each experimental day, all olfactometer parts (glass and Teflon tubes) were completely cleaned with tab water then distilled water and thereafter with acetone and pentane. After solvents had evaporated, the glass parts were placed overnight in an oven at 250 °C. The position of the odor source was changed clock-wise after each day of testing to avoid position effects. All bioassays were performed between 9:00 A.M. and 5:00 P.M. following D'Alessandro et al. [40].

2.4 Odor sources

2.4.1 Cowpea plants challenged by real herbivory

The following odor sources were offered to the parasitoids: (i) a cowpea plant primed with BTH and damaged by caterpillars, (ii) a cowpea plant primed with Laminarin and damaged by caterpillars and

(iii) a cowpea plant treated with distilled water and damaged by caterpillars. The remaining three vessels were left empty and alternated with vessels that contained the plants [44].

2.4.2 Cowpea plants challenged by mimicked herbivory

To rule out possible confounding effects of differences in HI-VOCs emissions due to differential damage inflicted by the caterpillars on plants with different treatments, we conducted an additional series of experiments with new sets of plants that were primed and then induced by mimicked herbivory, as described above. The odor sources offered in this experiment were: (i) a cowpea plant primed with BTH and induced by mimicked herbivory, (ii) a cowpea plant primed with Laminarin and induced by mimicked herbivory and (iii) a cowpea plant treated with distilled water and induced by mimicked herbivory. The three other vessels remained empty and were alternated with the treatment vessels.

2.5 Odor trapping and analysis

Volatiles emitted by the various odor sources from behavioral assay were trapped for 4 h during the bioassays on Super-Q adsorbent filters (25 mg, 80–100 mesh; Alltech Associates, Deerfield, Illinois, USA). Before use, the filters were washed with 3 ml dichloromethane and were then attached to the horizontal port at the top of each odor source vessel. Purified air entered the bottles at a rate of 1.1 L/min and air carrying the volatiles was pulled through each trap at a rate of 0.7 L/min (Analytical Research System, Gainesville, FL, USA). The rest of the airflow entered the choice chamber with the wasps. After each assay, the traps were extracted with 150 µL dichloromethane (Super solvent; Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µL dichloromethane were added to each sample as internal standards (IS). Samples were either analyzed immediately or stored at -80 °C (Supelco, Amber Vial, 7 mL with solid cap w/PTFE liner). Odor samples were analyzed using a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer (Agilent 5975C VL MSD). After injection of 2 µL of sample, the GC oven temperature was programmed at 40 °C for 3.5 min, and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min,

followed by a post-run of 5 min at 250°C before returning to initial conditions. Helium at constant flow (0.9 ml/min) was used as carrier gas. MS ionization was achieved by electron impact at emission current 30 µAmps in the ion trap held at 200 °C. MS data were collected in full scan mode with a mass range of m/z 40–300. Volatiles were identified by comparing their mass spectra with those of the NIST 05 library and by comparing their retention indices with those of previous analyses [19,40]. The total amount of VOCs emitted from each treatment was estimated as the sum of the amounts for all compounds released during the collection period (4 h), assuming equal ionization efficiency in the source of the mass spectrometer for the different compounds. It should be noted that this relative quantification does not provide the true ratios among the various VOCs in the emitted blend.

2.6 Plant inducers and leaf consumption by caterpillars

To test whether cowpea priming with either BTH or Laminarin had an impact on *S. littoralis* herbivory we measured the area of leaf consumed by the caterpillars. For this, leaves of the cowpea plants that were used as odor sources in the olfactometer bioassay under real herbivory were digitally scanned, and the missing areas were measured using ImageJ 1.48 (NIH, http://imagej.nih.gov/ij/) software. Each plant had been exposed to 10 *S. littoralis* larvae (2nd instar; n=18).

2.7 Statistical analysis

The functional relationship between parasitoid responses and the different volatile sources offered in the six-arm olfactometer was examined with a generalized linear model as described earlier [44]. The response of tested parasitoids (*n*= 6 parasitoids x 5 groups x 6 experimental days = 180 individuals) was statistically analyzed independently using a model that was fitted by maximum quasi-likelihood estimation in the software package R [45], and its adequacy was assessed through likelihood ratio statistics and examination of residuals. Before analysis, volatile data were log (ln) transformed to reduce heteroskedasticity and to approach normal distribution. One-way ANOVA (*F*-test) and Student-Newman-Keuls methods were then performed to compare means of emitted VOCs when the data

were normally distributed (Shapiro-Wilk test) and the variances were homogeneous (Levene test). In case those assumptions for normally distributed data with homogeneous variances could not be fulfilled, we used the non-parametric Kruskal–Wallis one-way analysis on ranks (H-test) and then compared treatment effects using Dunn's method. The same test was used to compare the leaf areas consumed by *S. littoralis* caterpillars in the feeding assay. These analyses were performed with SigmaPlot 12.3 (SPSS Inc, Chicago, IL, USA).

Because univariate analyses do not elucidate both qualitative and/or quantitative changes (composition) in released HI-VOC blends upon various treatments, a multivariate data analysis using PLS-DA (Partial Least Squares-Discriminant Analysis) was applied to the data, incorporating each compound as a variable and treatment as a factor [46]. PLS-DA can determine whether samples belonging to the different treatment groups can be separated based on either quantitative or qualitative differences in HI-VOCs. Two types of output were used: a matrix of 'scores' which display visually recognized sample structure separating treatment groups according to model components, and a matrix of 'loadings' which display the contribution of the variables (herein are the emitted HI-VOCs) to these components (PCs) and the relationships among the variables themselves. Furthermore, one important measure in PLS-DA is the variable importance in the projection (VIP) of each variable (HI-VOC). More specifically, variables that have VIP values > 1 are most effective into HI-VOCs blend [47]. In all cases, data were quantile normalized, cube root transformed and mean-centered and divided by the standard deviation of each variable before PLS-DA, using the comprehensive online tool suite MetaboAnalyst 3.0 [48].

3. RESULTS

3.1 Wasp behavior

3.1.1 Cowpea plants subjected to real herbivory

Here, we investigated whether foliar application of BTH and Laminarin alters the attractiveness of *S. littoralis*-infested cowpea plants to parasitoids. Generally, naïve female wasps of all three species preferred arms that carried the odor of *S. littoralis*-infested plants to empty arms of the olfactometer (Fig. 2i). *Cotesia marginiventris* females showed a strong preference for both BTH and Laminarin primed plants ($F_{3, 176}$=26.75; P<0.001) (Fig. 2.2.i), while *C. sonorensis* ($F_{3, 176}$=15.01; P<0.001) and *M. rufiventris* ($F_{3, 176}$=38.98; P<0.001) preferred the odor of BTH primed plants, but did not distinguish between untreated and Laminarin treated plants (Fig. 2.1.i, 2.3.i).

3.1.2 Cowpea plants subjected to mimicked herbivory

For cowpea plants that were induced by mimicked herbivory, results were similar to those obtained with real herbivory. All tested parasitoid species were predominantly attracted to BTH-primed plants (Fig. 2.ii). *C. marginiventris* females showed a strong preference for BTH and Laminarin primed plants ($F_{3,176}$=23.34; P<0.001) (Fig. 2.2.ii). Females of *C. sonorensis* ($F_{3,176}$=18.59; P<0.001) were only attracted to BTH-primed plants and showed insignificant attraction to Laminarin-primed plants compared with control plants (Fig. 2.1.ii). However, unlike in previous experiments with plants exposed to real herbivory, there was no significant increase in the attraction of *M. rufiventris* females to BTH-primed plants when compared with control plants. In all cases, VOCs from plants (treated and control) were significantly more attractive than the air coming from empty arms (Fig. 2).

3.2 Volatile emissions

3.2.1 Cowpea plants subjected to real herbivory

The volatile analyses (Table 1) showed that BTH-primed plants emitted significantly higher amounts of green leaf volatiles (GLVs) *i.e.*: (*E*)-2-hexenal ($F_{2,16}$=15.882, *P* <0.001); (*Z*)-3-hexen-1-ol ($F_{2,26}$=15.357, *P* <0.001); 1-octen-3-ol ($F_{2,17}$=5.747, *P*=0.012) and (*Z*)-3-hexenyl acetate ($F_{2,42}$=4.870, *P*=0.013) compared to Laminarin or control plants. The emission of homoterpenes *i.e.*: (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) from inducer-primed plants was significantly increased compared with control plants (H_2=24.942, *P*=<0.001; $F_{2,11}$=10.627,

P=0.003, respectively). The same pattern ($F_{2,10}$=12.579, P=0.002) was also observed for the ketone 2-methyl-2-hepten-6-one (MHO). Unlike (*E*)-β-ocimene, which was emitted in significantly higher amounts ($F_{2,24}$=10.473, P=<0.001) from BTH-primed plants, a significant decrease in emission of the monoterpene (*S*)-linalool was observed from inducer-primed plants compared to control plants ($F_{2,19}$=31.206, P=<0.001), which was also the case for indole ($F_{2,36}$=27.473, P=<0.001), β-caryophyllene ($F_{2,18}$=11.811, P=<0.001) and (*E*)-β-farnesene ($F_{2,22}$=14.927, P=<0.001).

3.2.2 Cowpea plants subjected to mimicked herbivory

For the plants that were mechanically damaged and treated with caterpillar regurgitant (mimicked herbivory) we found similar, but less dramatic differences in volatile emissions as compared to the emission patterns of HI-VOCs after real herbivory (Table 1). The plants with mimicked herbivory were missing several of the HI-VOCs that were detected in the blends released by plants with real herbivory. For GLVs, BTH-primed plants released significantly higher amounts of (*Z*)-3-hexen-1-ol ($F_{2,17}$=3.722, P =0.046), 1-octen-3-ol ($F_{2,10}$=16.932, P <0.001) and (*Z*)-3-hexenyl acetate ($F_{2,9}$=4.786, P=0.038) compared with Laminarin or control plants. There were also significantly higher emissions of the homoterpenes (DMNT, $F_{2,35}$=5.849, P =0.006; TMTT, $F_{2,5}$=13.524, P =0.010) for plants primed with BTH, but not when they were treated with Laminarin. Again, BTH primed plants emitted significantly lower amounts of (*E*)-β-caryophyllene ($F_{2,18}$=11.811, P <0.001) compared to Laminarin treated or control plants.

The total emission of HI-VOCS was significantly higher for BTH-primed plants under mimicked herbivory, compared to control or Laminarin-primed plants. For real herbivory there was a similar but only marginally significant trend (Table 1). Overall, quantitative differences in VOC emission rates of different compounds were found for all four treatments suggesting that it would be possible for wasps to distinguish between them.

3.2.3 Multivariate analysis of volatile release

A PLS-DA analysis of HI-VOCs emitted from cowpea plants subjected to feeding by *S. littoralis* neonates resulted in a model with two predictive principal components (PCs), explaining 11 % and 11 % of the

total variance in HI-VOCs collection (Fig. 3a). The greatest loadings of PC1 were for (*S*)-linalool (0.49), indole (0.40), (*E*)-β-farnesene (0.35) and (*E*)-β-caryophyllene (0.25), whereas major loadings of PC2 were 1-octen-3-ol (0.37), (*E*)-2-hexenal (0.27), (*S*)-linalool (0.26) and DMNT (0.22) (Fig. 3b). The first component (PC1) separated the volatile blends based on the amount of emitted HI-VOCs caused by inducer treatment, exposing quantitative differences in emission rates. The second component (PC2) separated blends qualitatively, according to the proportion of certain compounds in the total blend. These discriminating compounds were the ones that had a VIP value higher than 1 (Fig S1a, b). In descending order of importance, the compounds contributing to PC1 were nonanal (1.85), (*S*)-linalool (1.83), decanal (1.63), α-pinene (1.19), indole (1.09), (*E*)-β-farnesene (1.07) and (*E*)-2-hexenal (1.03) (Fig S1a), whereas (*S*)-linalool (1.75), nonanal (1.73), decanal (1.54), indole (1.27), α-pinene (1.12), (*E*)-2-hexenal (1.07), (*cis*)-3-hexen-1-ol (1.06) and (*E*)-β-farnesene (1.02) were identified with a VIP value higher than 1 for PC2 (Fig S1b).

To gain more insight into the impact of plant inducers on VOC profiles, we analyzed VOCs that were released from cowpea plants treated with chemical inducers and then subjected to mimicked herbivory, in a separate model using PLS-DA (Fig 4). Similar to real herbivory, there were two significant PCs, explaining 12.8 % and 11.8 % of the total variance in HI-VOCs collection. The main loadings of PC1 were for DMNT (0.52), (*E*)-β-ocimene (0.43) and (*E*)-2-hexenal (0.30), whereas the key loadings of PC2 were (*E*)-β-ocimene (0.44), TMTT (0.42), decanal (0.36) and nonanal (0.32) (Fig. 4b). Furthermore, for PC1, six compounds [(*E*)-β-ocimene (1.99), (*cis*)-3-hexen-1-ol (1.74), DMNT (1.46), α-pinene (1.24), *E*-2-hexenal (1.17) and decanal (1.07)] had a high discriminatory power (Fig S2a), with a VIP value >1, implying a strong contribution to the separation between the treatments. The highly influential compounds for the separation in PC2, with VIP value higher than 1, were (*E*)-β-ocimene (1.80), (*Z*)-3-hexen-1-ol (1.56), DMNT (1.37), α-pinene (1.27), (*E*)-2-hexenal (1.11) and decanal (1.01), which thus contributed the most to explaining the variation in the model (Fig S2b).

3.3 Leaf consumption by *S. littoralis* caterpillars

Caterpillar consumption of plants was similar on all cowpea plants, regardless of whether the plants were treated with inducers or not (Over nearly 20 h, leaf consumption [cm ^{2}± SE] on BTH-primed plants: 0.26 ± 0.03; Laminarin-primed plants: 0.34 ±0.06; control plants: 0.26 ± 0.05). Although leaf consumption on Laminarin-primed plants was somewhat higher, no significant effect of either BTH or Laminarin treatment was found on the amount of leaf material ingested by *S. littoralis* caterpillar compared with the control treatment (H_2=0.0552, P=0.759).

4. DISCUSSION

Certain chemical compounds can trigger defense priming in plants, which results in a faster and stronger deployment of plant defences in the event of a future attack, even before plants actually experience an herbivore or pathogen [26]. This also applies to a plant's indirect defense, *i.e.* the release of HI-VOCs that attract the natural enemies of herbivores [27,28]. Phenotypic manipulation of VOCs by applying priming agents has received considerable research attention recently due to its potential for providing more benign agrochemicals to use in crop protection [11,18,39].

In the current study, we found that treating cowpea with two priming agents not only modulates the emission of VOCs upon either real or mimicked herbivory, but it also enhances the overall attraction of three parasitoid species. Specifically, our data show that cowpea treatment with BTH, and in few cases Laminarin, significantly increases the emission of certain key HI-VOCs such as DMNT, (*E*)- β-ocimene, (*Z*)-3-hexenyl acetate and MHO, whereas BTH treatment solely increases the emission of (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, decanal and TMTT under real herbivory (Table 1). The same was true, but to a lesser extent, for treated cowpea plants that were challenged later by mimicked herbivory. In contrast, treatment of cowpea plants with BTH and Laminarin was found to reduce the emission of other HI-VOCs such as the monoterpene (*S*)-linalool, the sesquiterpenes (*E*)-β-caryophyllene and (*E*)-β-farnesene, and the aromatic volatile indole.

Using the same plant strengtheners, we previously showed similar effects on parasitoid attraction in maize and cotton. In those cases, treatment with BTH and Laminarin also increased DMNT, TMTT and trans-ocimene in subsequently damaged plants and decreased emission of sesquiterpenes, monoterpenes and indole [12,17]. This is fully in agreement with our current results. Interestingly, these effects can also be obtained when utilizing the BTH and Laminarin as seed treatments [18].

Elevated emissions of VOCs after treatment have also been reported for several other systems. For example, treating plants with *cis*-jasmone, an oxylipin that is produced naturally by plants, led to a significant increase in the emission of defensive VOCs in cotton [49]; potato [50]; soybean [23] and sweet pepper [20]. Similarly, treatment with methyl jasmonate (MeJA), which simulates herbivory, causes plants to emit significantly more VOCs [19,51–53], particularly DMNT [31]. The increased emission of VOCs upon treatment with these compounds can be attributed to subsequent changes in the plant transcriptome which then increases the emission of VOCs [15,54]. Treating plants with plant strengtheners such as BTH and Laminarin is typically done to induce systemic acquired resistance (SAR) against pathogens, which is mediated by salicylic acid (SA) and results in the upregulation of the marker genes PR1 and PR5 [17,29]. This may led to a down-regulation of genes that are involved in the biosynthesis of JA [55] and thus interfere with volatile production [6,56]. Due to such negative cross talk between the SA and the JA pathways [7], it is likely that these inducers act as suppressors of the herbivore-induced JA response, thereby suppressing the production and release of certain VOCs. This may explain the decreased emissions that we observed for sesquiterpenes, certain monoterpenes and indole in the treated plants. Semiz et al. [31] reported that the application of carrageenans, which, similar to Laminarin, are obtained from algal extracts, significantly reduced the emission of most constitutively produced monoterpenes in two woody tree species. Again, this may be due to changes in defense hormone levels.

Our behavioral assays revealed that *C. sonorensis* and *M. rufiventris* were more attracted to BTH treated plants than to control or Laminarin treated plants. *C. marginiventris* was significantly

more attracted to plants treated with either Laminarin or BTH compared to the untreated plants (Fig. 2). This enhanced wasp attraction was mostly consistent for both real and mimicked herbivory. Enhanced wasp attraction after the induction of pathogen resistance or the SA pathway [17,29,30], followed by herbivory was already reported for maize [16]. It was also recently shown for Brassica plants challenged by the bacterial pathogen *Xanthomonas campestris* Dowson with simultaneous herbivory by *Pieris brassicae* L. caterpillars, these doubly infested plants were more attractive to the specialized parasitoid *Cotesia glomerata* L. than plants only infested by caterpillars [9]. This pathogen-mediated increase of parasitoid attraction may explain increases in parasitism rates observed for herbivore-infested plants that were also infected with pathogens [57,58]. These results seem to imply additive beneficial effects from treating plants with inducers of pathogen resistance.

Several of the above-mentioned studies, including ours, have shown a substantial increase in GLVs, in particular (Z)-3-hexen-1-ol, 1-octen-3-ol and (Z)-3-hexenyl acetate, after priming treatment. GLVs can play an important role in the attraction of natural enemies [56,59]. Indeed, we show that BTH-primed plants, under real or mimicked herbivory, released considerable amounts of (Z)-3-hexen-1-ol which is known to be attractive to parasitic wasps. For instance, Wei *et al.*[60] reported that many fabaceous plants, including cowpea, release (Z)-3-hexen-1-ol, which attracts parasitoids of Agromyzid leafminers. Furthermore, attraction and electrophysiological activity to (Z)-3-hexen-1-ol, from faba bean plants, have also been observed for the syrphid *Episyrphus balteatus* (De Geer) [61]. We also found that 1-octen-3-ol was emitted in significantly higher amounts from BTH-treated cowpea compared to either Laminarin or control plants. 1-Octen-3-ol is a characteristic volatile of legumes [62] that is known to attract lepidopteran pests [63], but also a biological control agent of powdery mildew [64]. Our results further indicate that cowpea plants emitted higher amounts of (Z)-3-hexenyl acetate upon treatment with BTH and these plants were the most attractive to the parasitoids. (Z)-3-hexenyl acetate has also been reported to attract parasitoids [14,65,66] and is released from several plants after treatment with plant activators or phytohormones [66,67].

Various studies have reported a special role for 6-methyl-5-hepten-2-one (MHO), which was notably increased upon inducer treatment, in attracting parasitic wasps [68]. MHO is a specific semiochemical target for exploitation in the control of legume pests [69]. The two homoterpenes are also of special interest. It has been shown that DMNT [70,71] and TMTT [72,73] are essential HI-VOCs for attraction of natural enemies. Their emissions are strongly upregulated after plant treatment with resistance inducers or phytohormones [12,49,74].

Contrary to the above compounds, several typical HI-VOCs were emitted in considerably lower levels after treatment with BTH or Laminarin, in particular (S) linalool, (E)-β-farnesene, (E)-β-caryophyllene, and indole. This may also have importantly contributed to the enhanced parasitoid attraction [16,17]. Terpenoids are often assumed to be attractants for natural enemies, but, for instance, the transformation of *Arabidopsis* with typical terpene-synthase genes from maize revealed that the transformed lines were only attractive to parasitoids after the wasps learned to associate the terpenoid blend with the presence of caterpillar hosts [75,76]. For innate attraction these compounds are apparently not essential [40]. Their toxic effects [6] may be one reason for parasitoids to avoid them. This is particularly true for indole, which despite the fact that it is a very common HI-VOC, it appears not to be attractive and even repellent to parasitoids, especially *M. rufiventris* [77]. Indole is toxic to *Spodoptera* caterpillars [78] and possibly affects their suitability as hosts for parasitoids.

Insects probably perceive HI-VOCs as a blend that may vary in quantity and composition (quality) [46,79,80]. We therefore analyzed the volatile data using a multivariate tool (PLS-DA), allowing us to identify which HI-VOC combinations were associated with parasitoid preference. In all olfactometer observations, *Campoletis* and *Microplitis* females were most attracted to herbivore-damaged plants that had first been primed with BTH. The multivariate analysis revealed that BTH treated plants emitted a blend with reduced relative quantities of certain mono and sesquiterpene HI-VOCs [*i.e.*: (S)-linalool, (E)-β-farnesene], as well as less of the aromatic indole compared to either control or Laminarin treated plants. This was different for the monoterpene α-pinene, which emission was significantly increased after BTH and Laminarin treatments, respectively (Fig. S1, S2), and is known to

attract parasitoids [81]. Consistent to these findings, treated plants with JA or MeJA emit larger amounts of α- pinene, increasing the plants attractiveness to natural enemies [13,82].

The PLS-DA provided further hints at which compounds may be of importance for attraction. For instance, the relative concentrations of nonanal was higher in HI-VOCS blends from BTH-primed plants, as compared to control plants or Laminarin treated plants. That BTH treated cowpea release larger amounts of nonanal may be explained by antimicrobial properties of this compound [83], which corresponds with the more robust induction of resistance to many phytopathogens upon BTH treatment compared to Laminarin treatment [29,30]. Yu *et al.* [84] showed that the antennae of the parasitoid *Microplitis mediator* (Haliday) are highly responsive to nonanal released from caterpillar-damaged cotton plants. Similarly, *Cotesia plutellae* (Kurdjumov), a parasitoid of the diamondback moth, and the ectoparasitoid *Bracon hebetor* (Say), which parasitizes mealmoth larvae, show strong electroantennogram (EAG) responses and attraction to nonanal [85,86]. It should also be noted that nonanal baited traps in cotton fields are very attractive to predatory insects such as the anthocorid *Orius similis* Zheng and the syrphid fly *Paragus quadrifasciatus* Meigen [14].

The PLS-DA of induced HI-VOCs blend by mimicked herbivory further revealed that the relative concentrations of (*E*)-β-ocimene and DMNT were notably higher into the emitted volatile blend from BTH and Laminarin-primed plants compared to control cowpea plants (Fig S2), suggesting a possible involvement in the enhanced attraction of female parasitoids. (*E*)-β-ocimene is a common HI-VOC released from many plants in response to herbivory [87], and it plays an important role in the attraction of predatory mites [72,74] and aphid parasitoids [88]. The emission of (*E*)-β-ocimene can be induced by exogenous application of chemical elicitors and phytohormones or their analogues such as BTH [17], *cis*-jasmone [23,89], MeJA [51,53], and JA [15,74], and in each case this was found to enhance the attractiveness of the plants to the studied natural enemies. DMNT is also a very common inducible plant volatile that is used by various natural enemies to locate prey or hosts [12,70,71,74]. Interestingly, the antennae of *C. sonorensis* are highly responsive to DMNT [90], which might explain its strongly enhanced attraction to Laminarin-primed plants that were subjected to mimicked herbivory, which released DMNT in a

relative concentration that was considerably higher than the control or BTH treatments (Fig S2). In addition to VIP compounds, PLSDA of emitted HI-VOCS further revealed that, (*S*)-linalool and 1-octen-3-ol from plants with real herbivore damage, as well as DMNT and (*E*)-β-ocimene from plants with mimicked herbivory, had the greatest loadings for both PC 1 & PC 2. This suggests that the production of these compounds may be linked to wasp attraction (Fig 3b, 4b).

Treatment with BTH or Laminarin had no measurable impact on the leaf areas consumed by the caterpillars, but it should be noted that the *Spodoptera* caterpillars were only on the plants for 24 h. Rostás and Turlings [16] found the same for maize plants treatment with BTH, with no effect on the amount of damage done to leaves by *S. littoralis* caterpillars. This is different for tomato plants treated with BTH, on which growth and survival of *S. littoralis* larvae is significantly reduced [42]. In contrast, Gordy et al. [91] found that BTH treatment of cotton and soybean increased the growth rate of *Spodoptera frugiperda.* Various other studies showed a negative impact of plant treatments with inducers on the growth and performance of either sucking [92–95] or chewing [25,96,97] herbivores. This corresponds well with metabolic changes and activation of defense proteins that decrease the plants' nutritional quality to the feeding herbivores [97–99].

It has been proposed that plant inducers can be used to manipulate plant VOCs in order to enhance their attractiveness to natural enemies of pests [18,39,100]. The current findings further support the potential of this new and ecologically sound strategy, showing that it is possible to use BTH or Laminarin treatments to alter HI-VOC emission in cowpea and increase parasitoid attraction. Yet, field experiments are still required to confirm that such treatments lead to increased parasitism of pests under realistic conditions. Given the fact that intercropping with grain legumes is currently seen as a possible mechanism for vertical intensification in modern agriculture [101] and the high nutritional value of cowpea as a main source of protein for low income populations in tropical regions [33,34], any improvements to plant performance and yield would be highly valuable. The results of our current study may prompt further steps towards a novel strategy for the control of deleterious lepidopteran insect pests that attack this and other key legume crops.

ACKNOWLEDGEMENTS

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Figures Captions

Fig 1. Schematic representation shows the experimental procedure of the HI-VOCs sampling from cowpea plants and wasp response to these plants. (A) HI-VOCs collection from cowpea plants that were induced using real herbivory by adding 10 larvae (2nd instar) of *Spodoptera littoralis* to each cowpea plant. (B) HI-VOCs collection from cowpea plants that were induced using mimicked herbivory in which two leaves of each cowpea plant were wounded and then treated with 20µl of *S. littoralis* oral secretions (3x dil.). VOCs sampling was carried out for 4 h for both real and mimicked herbivory.

Fig 2. Response and preference of naïve adult females of three different endoparasitoids tested in a six-arm olfactometer. Values shown are the number of parasitoid females choosing a particular odor. Parasitoids were allowed to choose between odors: BTH = BTH-treated cowpea plants; Laminarin= laminarin-treated cowpea plants; Control= cowpea plants treated with distilled water; Empty=empty control vessel (mean value of three vessels). (i) Ten 2nd instar larvae of *Spodoptera littoralis* were added to plants 24 h before the experiment. (ii) Plants were mechanically damaged and then treated with *S. littoralis* oral secretions. Different letters above bars indicate significant differences between treatments (P < 0.05, *F*-test).

Fig 3. PLS-DA comparison of the volatile compounds emitted by cowpea plants (15 d-old with 4-5 fully developed leaves) sampled for 4 h. Plants (n=18) were challenged by ten 2nd instar larvae of *Spodoptera littoralis*. BTH = BTH-treated cowpea plants (●); Laminarin= laminarin-treated cowpea plants (●); Control= cowpea plants treated with distilled water (●). (a) Score plot showing the ordination of the samples according to the first two PLS components based on the quantitative values of HI-VOCs between different treatments, with the percentage of explained variation in parentheses. The PLS-DA resulted in a model with one significant component: R^2X=0.2167, R^2Y=0.2647, Q^2=0.296. (b) Loading plot shows the contribution of each HI-VOC to the discrimination between treatments

using the first two PLS components. Numbers refer to the volatile compounds listed in Table 1. The ellipses display 95 % confidence regions.

Fig 4. PLS-DA comparison of the volatile compounds emitted by cowpea plants (15 d-old with 4-5 fully developed leaves) sampled for 4 h. Plants (n=18) were mechanically damaged and then treated with *S. littoralis* oral secretions. BTH = BTH-sprayed cowpea plants (●); Laminarin= laminarin-sprayed cowpea plants (●); Control= distilled-water-sprayed cowpea plants (●). (a) Score plot showing the ordination of the samples according to the first two PLS components based on the quantitative values of HI-VOCs between different treatments, with the percentage of explained variation in parentheses. The PLS-DA resulted in a model with one significant component: R^2X=0.186, R^2Y=0.226, Q^2=0.328. (b) Loading plot shows the contribution of each HI-VOC to the discrimination between treatments using the first two PLS components. Numbers refer to the volatile compounds listed in Table 1. The ellipses display 95 % confidence regions.

Table 1. Mean amount of VOCs (in ng; Mean ± SE; n = 18) emitted by cowpea plants treated with BTH, Laminarin and distilled water (control) over 4 h collections carried out in a six-arm olfactometer. Plants were induced by either **(i)** Real herbivory: challenging plants with ten 2nd instar larvae of *Spodoptera littoralis* or **(ii)** Mimicked herbivory: wounding plants and then adding *S. littoralis* regurgitant, 24 h before the experiment.

Compounds		(i) Real herbivory-damaged plants				(ii) Mimicked herbivory-damaged plants			
		BTH	Laminarin	Control	*P* value	BTH	Laminarin	Control	*P* value
1	(*Z*)-3-hexenal [C/GLV]	30.5±1.7	31.9±3.9	31.1±4.3	*P*=0.881	ND	ND	ND	--
2	(*E*)-2-hexenal[C/GLV]	40.3±3.2^a	20.2±3.5 ^{b}	13.3±3.6^b	**P<0.001**	44.8±7.5	28.2±4.4	38.8±7.2	*P*=0.221
3	(*Z*)-3-hexen-1-ol[C/GLV]	47.9±4.7^a	28.7±2.6^b	22.7±1.8^b	**P<0.001**	48.4±10.4^a	21.1±2.6^b	20.9±3.4^b	**P=0.046**
4	*α*-pinene[C/MT]	56.1±10.6	58.3±20.7	51.4±17.9	*P*=0.826	69.7±24.5	66.5±17.4	49.7±13.6	*P*=0.877
5	MHO[C/KT]	31.8±3.9^a	29.3±4.1^a	10.8±2.7^b	**P=0.002**	23.7±6.5	15.1±1.1	6.4±5.3	*P*=0.203
6	1-octen-3-ol[C/ GLV]	43.4±4.7^a	19.2±4.9^b	19.6±4.4^b	**P=0.012**	32.1±3.6^a	15.6±1.8^b	10.1±2.4^b	**P<0.001**
7	Myrcene[C/MT]	19.9±4.3	21.8±6.4	17.1±4.7	*P*=0.771	54.4±16.6	45.4±5.4	45.1±17.6	*P*=0.965
8	(*Z*)-3-hexenyl acetate[I/GLV]	54.8±9.1^a	51.8±8.7^a	27.3±2.9^b	**P=0.013**	62.3±22.9^a	24.4±7.9^{ab}	14.6±4.8^b	**P=0.038**
9	(*E*)-*β*-ocimene [I/MT]	87.4±9.9^a	63.6±15.5^a	24.7±4.8^b	**P<0.001**	45.3±19.2	20.6±3.1	10.1±2.9	*P*=0.115
10	Nonanal[C/ ALH]	13.2±1.9	14.3±4.1	13.8±6.1	*P*=0.882	31.3±2.8	28.5±4.1	31.8±3.1	*P*=0.690
11	(*S*) linalool [I/MT]	14.1±3.8^b	21.4±1.7^b	70.7±7.8^a	**P<0.001**	ND	ND	ND	--
12	DMNT [I/HT]	296.1±24.6^a	208.9±21.2^a	101.2±11.4^b	**P<0.001**	218.8±36.6^a	103.7±20.9^b	70.8±14.7^b	**P=0.006**
13	Decanal[C/ALH]	36.1±11.4^a	15.2±1.7^b	15.1±2.2^b	**P=0.002**	25.5±3.6	27.6±5.2	25.4±5.3	*P*=0.704
14	Indole [I/AR]	27.9±3.9^b	40.1±5.2^b	126.8±19.1^a	**P<0.001**	ND	ND	ND	--
15	(*E*)-*β*-caryophyllene [C/ST]	10.4±1.7^b	22.9±2.9^b	43.8±7.3^a	**P<0.001**	20.3±5.6^b	38.1±19.6^a	67.5±5.9^a	**P=0.013**
16	(*E*)-*β*-farnesene [I/ST]	20.4±3.9^b	27.1±6.4^b	82.5±11.8^a	**P<0.001**	ND	ND	ND	--
17	Nerolidol [C/ST]	25.9±4.5	32.8±1.9	31.6±7.8	*P*=0.577	ND	ND	ND	--
18	TMTT [I/ HT]	154.8±19.1^a	103.8±10.3^b	70.8±6.2^b	**P=0.003**	102.1±12.8^a	34.1±4.6^b	41.3±6.26 ^{b}	**P=0.010**
19	Total HI-VOCs	560.1±53.3	448.9±67.1	433.7±43.3	*P*=0.073	350.9±63.5^a	208.7±35.6^{ab}	177.5±31.1^b	**P=0.045**

The compounds are ordered in accordance with their increasing retention time in a gas chromatograph. A natural log (ln) transformed values were analyzed using one way analysis of variance. Different letters indicate significant differences between treatments (P < 0.05). *P* values in bold indicate significant difference. ([ALH:] Aldehyde; [AR:] Aromatics; [C:] Constitutive compounds; DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene, [GLV:] Green Leafy Volatiles; [HT:] Homoterpene; [I:] Inducible Compounds; [JD:] Jasmonate derivative; [KT:] Ketone, MHO: 2-methyl-2-hepten-6-on, [MT:] Monoterpene; ND: compound not detectable; [ST:] Sesquiterpene; TMTT: (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene). The absolute configuration of chiral volatiles is not determined.
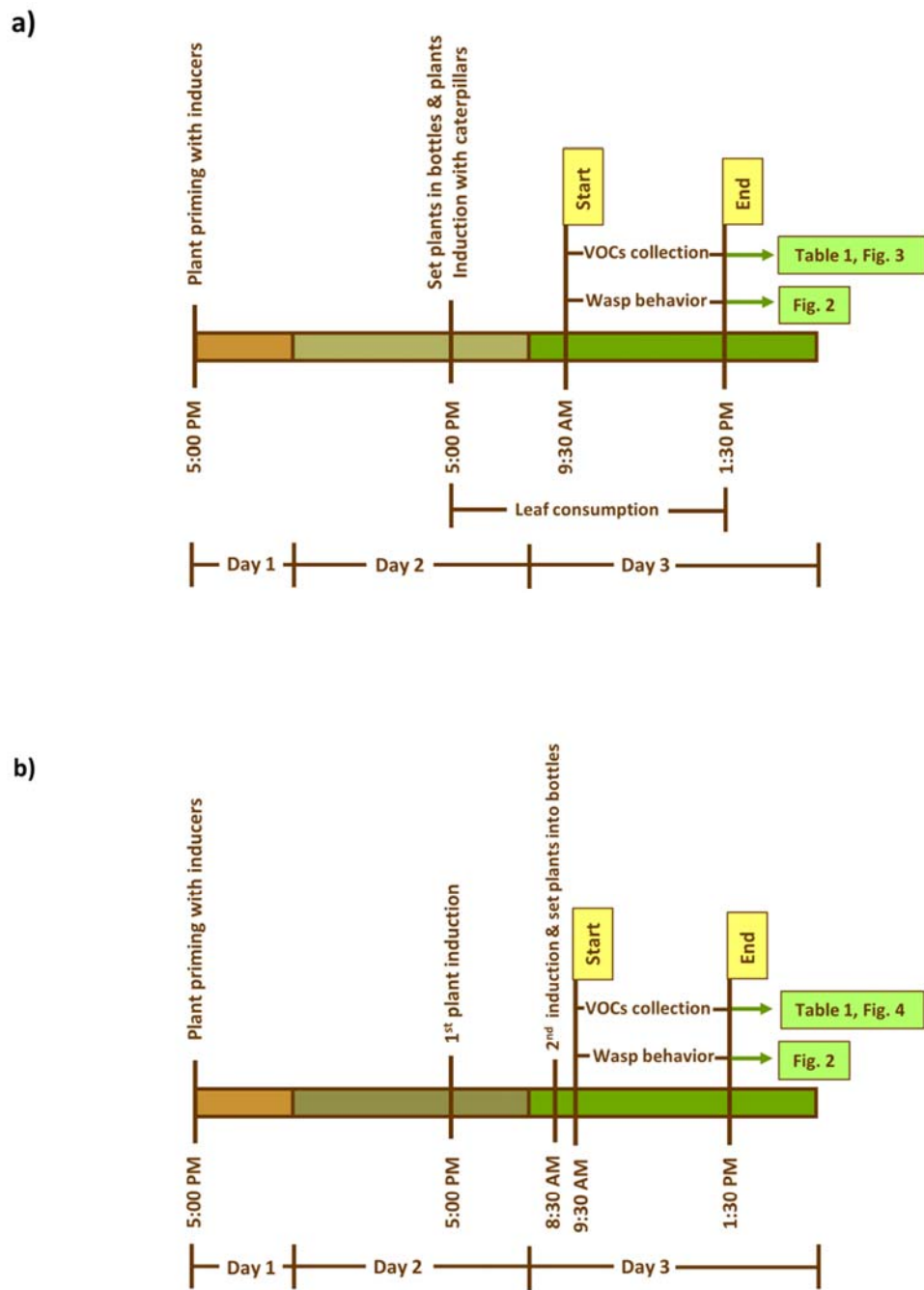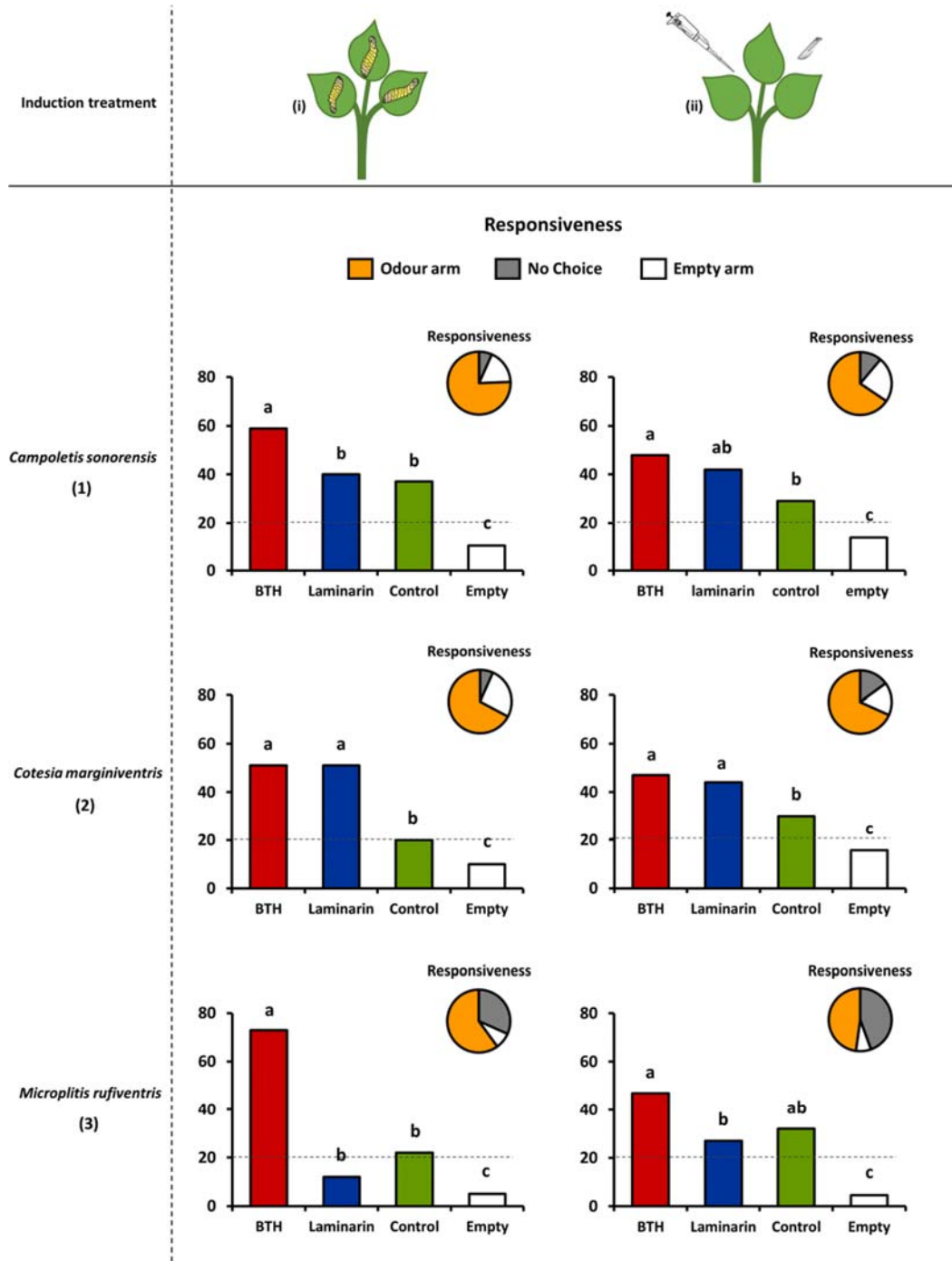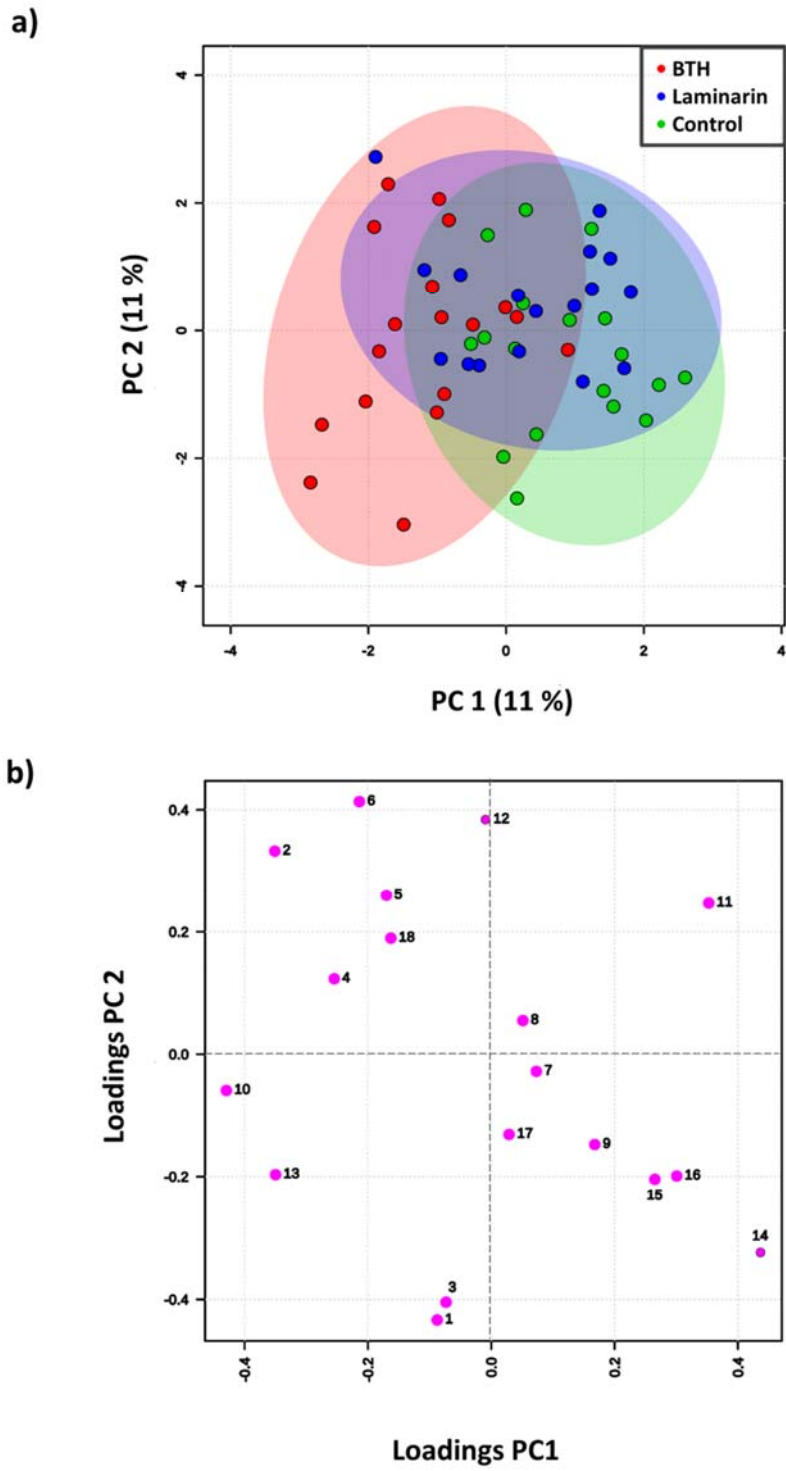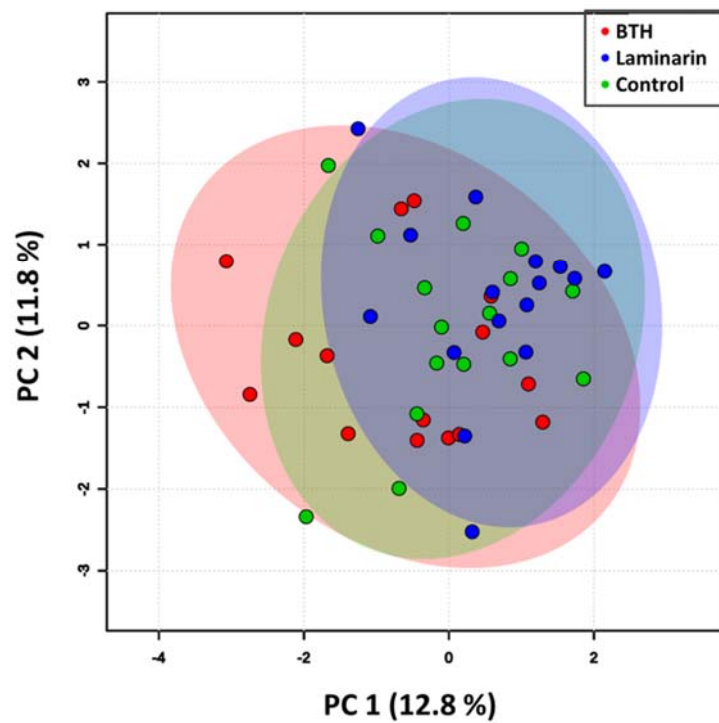
a)

Plant priming with inducers

Set plants in bottles & plants
Induction with caterpillars

Start

End

VOCs collection → Table 1, Fig. 3

Wasp behavior → Fig. 2

5:00 PM   5:00 PM   9:30 AM   1:30 PM

Leaf consumption

Day 1   Day 2   Day 3

b)

Plant priming with inducers

1st plant induction

2nd induction & set plants into bottles

Start

End

VOCs collection → Table 1, Fig. 4

Wasp behavior → Fig. 2

5:00 PM   5:00 PM   8:30 AM 9:30 AM   1:30 PM

Day 1   Day 2   Day 3

Fig. 1

Fig. 2

a)

b)

Fig 3

a)

b)

Fig 4