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Priming Seeds with Indole and (*Z*)-3-Hexenyl Acetate Enhances Resistance Against Herbivores and Stimulates Growth

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Abstract

A striking feature of plant ecology is the ability of plants to detect and respond to environmental cues such as herbivore-induced plant volatiles (HIPVs) by priming or directly activating defenses against future herbivores. However, whether seeds also respond to compounds that are common constituents of HIPV blends and initiate future plant resistance is unknown. Considering that seeds depend on other environmental cues to determine basic survival traits such as germination timing, we predicted that seeds exposed to synthetic constituents of HIPV blends would generate well-defended plants. We investigated the effect of seed exposure to common volatiles on growth, reproduction, and resistance characteristics in the model plants *Arabidopsis thaliana* and *Medicago truncatula* using herbivores from two feeding guilds. After seed scarification and vernalization, we treated seeds with one of seven different plant-derived volatile compounds for 24 h. Seeds were then germinated and the resulting plants were assayed for growth, herbivore resistance, and expression of inducible defense genes. Of all the synthetic volatiles tested, indole specifically reduced both beet armyworm growth on *A. thaliana* and pea aphid fecundity on *M. truncatula*. The induction of defense genes was not affected by seed exposure to indole in either plant species, indicating that activation of direct resistance rather than inducible resistance is the mechanism by which seed priming operates. Moreover, neither plant species showed any negative effect of seed exposure to any synthetic volatile on vegetative and reproductive growth. Rather, *M. truncatula* plants derived from seeds exposed to (*Z*)-3-hexanol and (*Z*)-3-hexenyl acetate grew larger compared to controls. Our results indicate that seeds are sensitive to specific volatiles in ways that enhance resistance profiles with no apparent costs in terms of growth. Seed priming by HIPVs may represent a novel ecological mechanism of plant-to-plant interactions, with broad potential applications in agriculture and seed conservation.

Keywords Aphid · Beet armyworm · Herbivore-induced plant volatiles (HIPVs) · Indole · Plant defense · Plant growth · Plant volatiles · Plant communication · Seed priming

Introduction

Spermatophytes (or seed plants) are a dominant clade of vascular plants on earth (Friis et al. 2011; Simonin and Roddy 2018). Their dominance is due in large part to the evolution of the seed, which provides protection to the embryo prior to germination and nutrition during the transition to autotrophy. The seed is typified by developmental stages intended to maximize germination success. One advantage of the seed is the ability to survive long periods in dormancy (Finch-Savage and Leubner-Metzger 2006), which is defined as the inability of a viable seed to germinate under favorable environmental conditions (Holdsworth et al. 2008). Dormancy can occur during seed maturation (primary), which does not occur in all plant species, or later induced (secondary) (Buijs et al. 2020). Primary dormancy is overcome via "after-ripening", a time- and environment-sensitive process allowing the capacity for seeds to complete germination when exposed to favorable germination conditions (Chahtane et al. 2016). Even once dormancy is broken, non-dormant seeds can remain ungerminated until environmental conditions are suitable for germination and

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growth (Carrera et al. 2008). During the complex developmental stages prior to germination, seeds are inevitably exposed to a variety of biotic and abiotic environmental conditions such as temperature, moisture, fire, soil chemicals, and chemical exudates of plant and microbial origin that may affect their germination (Fenner 2000). Many of these conditions are well-established cues that seeds use to coordinate their physiology and metabolism to properly time germination to maximize viability and establishment (Bentsink and Koornneef 2008; Koornneef et al. 2002). Temperature (Probert 2000; Reynolds et al. 2001), rainfall (Gutterman 1994; Levine et al. 2008; Pake and Venable 1996), and light (Flores et al. 2006; Milberg et al. 2000; Wesson and Wareing 1969) are well-documented abiotic environmental cues that affect the germination of seeds, and responses to these cues are regulated through phytohormone signaling pathways (Chen et al. 2008; Seo et al. 2009; Toh et al. 2008).

In addition to abiotic cues, seeds can perceive a variety of chemical cues of biological origin that can affect germination and subsequent defensive profiles. For example, low molecular weight phenolic compounds in soil (Muscolo et al. 2001), artemisinin released from leaves (Chen and Leather 1990) and catechin released from plants after herbivory (Thelen et al. 2005) inhibit seed germination. In contrast, smoke-derived karrikins (Dixon et al. 2009; Flematti et al. 2004; Nelson et al. 2012) and strigolactone (SL) phytohormones released from plant roots can stimulate seed germination (Bergmann et al. 1993; Cook et al. 1966). Moreover, the direct application of exogenous phytohormones to seeds can activate plant defenses (Haas et al. 2018; Rajjou et al. 2006; Worrall et al. 2012). For example, treating tomato seeds with jasmonic acid (JA) and β-aminobutyric acid (BABA) led to plants expressing JA- and ethylene-dependent resistance against spider mite, caterpillars, aphids, and pathogens (Worrall et al. 2012). Seed treatment with JA also changes the volatile composition of the subsequent plants, making their blends more attractive to predatory mites (Smart et al. 2013). Similarly, seed treatment with salicylic acid (SA) enhances the expression of SA-related genes and the endogenous SA level against root holoparasite (*Orobanche cumana*) (Yang et al. 2016). Additionally, seed coating with plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungus (PGPF) enhances seed germination, seedling establishment, and boosts induced defenses in future plants in SA-, ET-, and JA-dependent manners (Rudrappa et al. 2010; Ryu et al. 2004; Sharifi and Ryu 2016).

Seeds also come in contact with volatile biotic agents that can also provide informational cues or directly affect germination. Inhibitory and allelopathic effects of some plant and microbial-derived volatile organic compounds (VOCs) have been known for a long time (Bradow and Connick 1990; Koitabashi et al. 1997; Mirabella et al. 2008; Muller 1965; Muller and Muller 1964; Oleszek 1987). Whereas these VOCs do not necessarily provide contextual information about future environmental conditions, herbivore-induced plant volatiles (HIPVs) represent potentially reliable and adaptive indicators of herbivory (Frost et al. 2008a). The function of HIPVs in priming or directly inducing plant defenses is now well established (Engelberth et al. 2004; Frost et al. 2007; Rodriguez-Saona and Frost 2010), and exposure of undamaged plants to HIPVs is known to induce or prime the genes in phytohormone pathways (Bate and Rothstein 1998; Engelberth et al. 2007; Frost et al. 2008c). Moreover, aboveground HIPV priming cues are also produced belowground by plant roots (Barsics et al. 2017; Gfeller et al. 2013; Lawo et al. 2011; Palma et al. 2012) and rhizosphere organisms (Bhattacharyya et al. 2015; Kanchiswamy et al. 2015). There are multiple ecologically relevant routes by which seeds could be exposed to HIPVs, including simple diffusion of HIPVs produced belowground (Peñuelas et al. 2014) and precipitation and leaching of HIPVs produced aboveground (Muller et al. 1964; Tukey Jr 1970). While some HIPVs may have allelopathic effects on seed germination (Karban 2007; Mirabella et al. 2008; Preston et al. 2001), whether exposure of seeds to HIPVs alters subsequent plant physiology and resistance is currently unknown.

Here, we tested the hypothesis that post-dormancy, ungerminated (“activated”) seeds respond to the application of exogenous, individual synthetic volatiles that are common constituents of HIPV blends and determined the effect of seed exposure to these volatiles on plant growth, resistance against herbivores, and direct defense genes. Specifically, we used a comparative approach to investigate the effects of volatile exposure to the seeds of (1) *A. thaliana* on the performance of a chewing herbivore (beet armyworm; *Spodoptera exigua)* and (2) *M. truncatula* on the performance of a phloem-feeding herbivore (pea aphid; *Acyrthosiphon pisum*). We also tested the effect of seed exposure to plant volatile on the growth, development, and inducible defense gene expression of *A. thaliana* and *M. truncatula*. We specifically tested volatiles that have been shown previously to prime plants: indole, (*Z*)-3-hexenol (*z*3HOL), (*Z*)-3-hexenyl acetate (*z*3HAC), β-caryophyllene (BCP), and (*E*)-2-hexanal (*e*2HAL) (Erb et al. 2015; Farag et al. 2005; Frank et al. 2021; Frost et al. 2008c; Mirabella et al. 2008; Scala et al. 2013). We predicted that volatile exposure to seeds would prime the resulting plants for enhanced resistance against both chewing and phloem-feeding herbivores.

Methods and materials

Plant Material. We posited that seeds in a non-dormant, ungerminated "activated" physiological state would be receptive to volatile cues, as it is this physiological state where environmental cues are critical to germination and germination can occur (Holdsworth et al. 2008). We, therefore, prepared the seeds for the post-dormancy developmental stage prior to radicle or plumule emergence or other outward signs of germination. This physiological stage can be mimicked experimentally by stratification (for both plant species) and scarification (for *M. truncatula*).

A. thaliana (Col-0) seeds were surface sterilized in 75% (v/v) ethanol for five minutes and 20% bleach (v/v) in 0.01% Tween-20 for ten minutes. After sterilization, the seeds were washed three times with distilled water and spread on petri-plates with wet Whatman paper. Seeds were then stratified on petri plates kept at 4 °C for 2 days.

M. truncatula, A-17 seeds were scarified in concentrated H_2SO_4 for 10 min and surface sterilized in 20% (v/v) bleach in 0.1% (v/v) Tween-20 solution for 10 min. Seeds were rinsed five times with sterile water and were spread on petri plates with wet Whatman paper. Petri plates were covered with aluminum foil and kept at 4 °C for two days.

Seed Treatment with Plant Volatiles. Volatile dispensers were used to treat *A. thaliana* and *M. truncatula* seeds to individual plant volatiles. For preparing volatile dispensers 20 µl of *z*3HOL, *z*3HAC (Engelberth et al. 2004), *e*2HAL, β-caryophyllene, and 20 mg indole (Erb et al. 2015) was added into separate 2.0 ml amber glass vial (Agilent Technologies) with 1 mg of glass wool (Figure S1). Indole is a solid at room temperature, but the masses of the different volatiles were similar based on specific density (1 µl = 1.17 mg). Control volatile dispensers had only glass wool. The amber vials (control or with volatiles) were sealed with a rubber septum and connected to the 2-ounce plastic cup by piercing the plastic cup and amber vial rubber septum with an 18-gauge needle. This procedure of using a volatile delivery system with a restricted diameter connection between the volatile and the seeds was similar to what has been used previously for controlled administration of indole to plants (Erb et al. 2015), except that there was no airflow in our treatment to seeds. Whereas priming experiments on aboveground plant organs often appropriately use artificial air flow to mimic natural air movement (e.g., Frost et al. 2007; Frost et al. 2008c), seeds often are in soil environments with limited air flow and where volatiles may concentrate and not disperse long distances (e.g., β-Caryophyllene in Rasmann et al. (2005)) and static volatile delivery systems are common experimental techniques for seed treatments (Bradow and Connick 1990; Koitabashi et al. 1997; Mirabella et al. 2008; Muller 1965; Muller and Muller 1964; Oleszek 1987). Each volatile was administered to seeds in multiple plastic cups (biological replicates) and seeds planted from within a single plastic cups were considered technical replicates of each other. No visible signs of germination were observed during volatile exposure treatments.

A. thaliana Seed Germination and Growth. After one day of volatile treatment, two *A. thaliana* seeds were transferred from each plastic cups to agar plates containing 1.0% (w/v) agar (Sigma) and standard 0.5X MS medium (Murashige and Skoog basal salts, pH of 7.0). Nine agar plates (units of replication) with ten seeds each were used for each volatile treatment. Percent germination on each plate was determined by the number of seeds that germinated on the plate. The Petri dishes were kept in a growth chamber at 25 °C under a 16 h light: 8 h dark (16 L: 8D) day/night cycle for two days. Percent seed germination was measured after two days of seed transfer from plastic cup to petri-plates.

After one day of volatile treatment, *A. thaliana* seeds were transferred to 5.5 × 5.5 × 5.5 cm pots filled with sterile Metro-Mix 360 soil. After transplanting, pots were placed on trays (54 × 28 × 6 cm) in a growth chamber at 25 °C under a 12 h light: 12 h dark (12 L: 12D) cycle (Conviron). Once seedlings had 4–6 leaves, they were fertilized twice a week with 10 ml 1/2 strength Hoagland's solution. Arabidopsis growth and fitness were measured in terms of number of leaves, maximum rosette diameter, the length of the bolt, and number of siliques produced. This experiment included 58 plants, with 8–10 replicates per treatment.

M. truncatula Growth. Volatile-exposed *M. truncatula* seeds were planted in 9 × 6.5 × 6.5 cm pots. The trays were kept in growth chamber at 25 °C under a 12 h light: 12 h dark (12 L: 12D) day/night cycle for ten days. After 10 days the trays were moved to greenhouse and kept there until the end of the experiment. *M. truncatula* growth and fitness were measured in terms of petiole length, leaf blade length, leaf blade width, main shoot length, axillary shoot length, and number of fruits using a numerical nomenclature coding system developed by Bucciarelli et al. (2006). The numerical nomenclature for vegetative growth (see Fig. 3B) starts with first unifoliate leaf as metamer 1 (m1) followed by first trifoliate as metamer 2 (m2) and so continues likewise. The axillary shoots are coded from their metamer of origin (e.g., the axillary shoot originating from first unifoliate or metamer 1 is also designated as m1). Additionally, decimal addition to numerical coding system defines the development stage of the leaf (e.g., m2.1 represents the bud break for the first trifoliate, m2.5 represent the half-open blade of first trifoliate while m2.9 represents fully developed first trifoliate). Sixty plants were included in this experiment, 10 per treatment group.

Caterpillar Herbivory. Beet armyworm (*S. exigua*) was used to evaluate the effect of seed exposure to HIPVs on

Arabidopsis resistance against caterpillar herbivory. Egg masses were ordered from Benzon Research Inc. USA (Permit #P526P-16-02563, as required for interstate transport of *S. exigua* at the time of our study), and were immediately transferred to artificial diet in 2-ounce plastic cups, then maintained at 24 ºC on artificial diet until the desired instar. Third instar caterpillars were used for feeding experiments on five to six-week-old, vegetative stage, Arabidopsis plants. For the first feeding experiments, caterpillars were first starved for 3 h and weighed before their transfer to Arabidopsis plants. One third-instar caterpillar was placed on a single Arabidopsis plant. The plants were covered with a nylon mesh bag to contain the caterpillars. The caterpillars were allowed to feed freely for 24 h before being removed and kept at room temperature for three hours before being re-weighed. This experiment was repeated three times, the first two trials included all five volatiles, and the last trial focused only on indole. Trial 1 included 48 plants with 6–8 biological replicates per treatment. Trial 2 included 100 plants with 6 biological replicates per treatment (different treatment cups) and 2–3 technical replicates per biological replicate (seeds derived from the same treatment cup). Trial 3 included 43 plants, 17 controls (8 biological replicates with 1–3 technical replicates each), and 26 indole-treated (10 biological replicates with 1–4 technical replicates each). Aboveground plant material was collected in liquid nitrogen and stored at -80 ºC.

Aphid Herbivory. Pea aphid (*Acyrthosiphon pisum*) colony was maintained on fava bean plants in a growth chamber (20 ºC, 12:12 h light:dark). For aphid feeding experiments, three adult aphids (defined as the F_0 generation) (Tomczak and Müller 2017) were placed in a mesh bag (L15 X W6, BugDorm) on three trifoliates (6 to 8 plants per treatment; 43 plants in total). After 24 h, the adults were removed and one trifoliate was collected for molecular analysis, and 5 nymphs (defined as F_1 generation) were left on the plant for 13 more days. For 13 d the nymphs grew and produced offspring (F_2 generation). On day 14, all the aphids were collected and the total offspring (F_2) were counted and weighed collectively. The remaining trifoliates were also collected on day 14 into liquid nitrogen and stored at -80 ºC.

Gene Expression Analysis. Leaf tissue collected from *A. thaliana* plants after one day (24 h) of caterpillar herbivory and *M. truncatula* after 14 d of aphid feeding were used for gene expression analysis. We selected a 24 h time point for *S.exigua* herbivory on *A. thaliana* because both faster and stronger priming responses have been observed previously at this time point with real caterpillar herbivory (Frost et al. 2008c) and it coincided with the length of the caterpillar feeding trials. The time point for *M. truncatula* likewise coincided with the end of the aphid feeding trials, providing an opportunity to explore sustained effects of indole seed treatment on aphid-inducible marker genes (Maurya et al. 2018). Total RNA was isolated from approx. 150 mg of ground tissue using modified cetyl trimethylammonium bromide (CTAB) method (Frost et al. 2012). RNA was quantified with Nanodrop and integrity was confirmed using a native 1% agarose-0.5x TAE gel. Total RNA (2.5 µg per sample) was treated with DNAse (Turbo DNAse, Ambion), then 0.7 µg of DNA-free RNA was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcript Kit (Applied Biosystems). Real-time PCR was performed using the Quant Studio-3 PCR System (Applied Biosystems) with each reaction containing 2 µl of EvaGreen® PCR Master Mix (Mango Biotechnology), 0.3 µl of 10 µM forward and reverse primer, 5.4 µl of DI water, and 2 µl (2.5 ng) of cDNA in a total volume of 10 µl. Primer specificity was confirmed by melting curve analysis, and relative transcript levels were calculated using the $2^{-\Delta CT}$ method (Frost et al. 2012; Tsai et al. 2006) with Actin-7 and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reference genes for *A. thaliana*. and elongation factor 1-alpha (*EF1-α*) and *GAPDH* as reference genes for *M. truncatula*. Primer sequences for all genes tested are listed in Supplemental Table S1.

Statistical Analysis. Statistical analyses were performed using R version 4.0.3. Caterpillar growth rate was analyzed using a mixed model (lme4) with volatile treatment as a fixed factor and Experiment as a random factor. Dunnett contrasts (glht) assessed statistical comparisons between controls and each volatile treatment. Aphid fecundity and nymph weight were analyzed for significance using (glm) followed by Dunnett contrasts. For plant growth assays, leaf number and rosette diameter of *A. thaliana* were analyzed using mixed models (lme4) with Treatment and Time as fixed, interacting effects and Plant as a random factor. All other growth data for *A. thaliana* were analyzed with glm followed by Dunnett contrasts. *M. truncatula* growth data were analyzed with mixed models with Treatment and Metamer Code as fixed factors and Plant as a random factor. Subsequently, metamer-specific data were analyzed by glm followed by Dunnett contrasts. Gene expression data were analyzed using glm followed by Tukey's HSD contrasts to test for differences among all the treatment groups. Residuals of the models were checked for normality and homogeneity of variance. Figures were generated in R using ggplot2.

Results

Seed Exposure to Indole Enhances Plant Resistance against Chewing and Sap-feeding Herbivores. Indole exposure to *A. thaliana* seeds reduced the relative growth rate of *S. exigua* caterpillars feeding on subsequent plant foliage by an average of 37.1% ($P = 0.003$, Fig. 1 A). In contrast, seed exposure to the three GLVs and BCP had no consistent effect on *S. exigua* growth. Indole seed exposure had similar effects on pea aphids feeding on *M. trucatula*, reducing fecundity by an average of 28.0% on plants grown from indole-treated seeds relative to control seeds ($P = 0.022$, Fig. 1B). Additionally, *z*3HAC seed treatment to *M. truncatula* reduced pea aphid fecundity by 27.1% ($P = 0.037$, Fig. 1B). Total nymph weight was also reduced by an average of 40.6% in the indole seed-treated group relative to controls ($P = 0.038$, Fig. 1 C).

Seed Exposure to Indole does not Affect Growth and Development of A. thaliana. A. thaliana seed exposure to volatiles had no significant negative effect on the vegetative and reproductive growth relative to controls, and in fact exposure to z3HOL increased metrics of vegetative growth over time. We found Treatment*Time interactions for leaf counts (Treatment *Time $P = 0.015$, Fig. 2 A) and rosette diameter (Treatment*Time $P < 0.001$, Fig. 2B), both of which were the result of enhanced growth over time in plants grown from z3HOL-treated seeds relative to controls (Supplemental Fig. 2). There were no statistically significant differences in shoot mass ($P = 0.108$, Fig. 2 C), bolt length ($P = 0.334$, Fig. 2D), or silique number ($P = 0.460$, Fig. 2E) from seeds exposed to any volatile relative to control plants. There was a modest effect of z3HOL seed treatment on shoot fresh mass ($P = 0.089$).

We also measured the effect of volatile exposure on seed germination of *A. thaliana* on MS media. Indole did not affect seed germination and, of all the volatiles tested, only seed exposure to the GLV *e*2HAL reduced seed germination compared to controls ($P < 0.001$, Fig. 2 F).

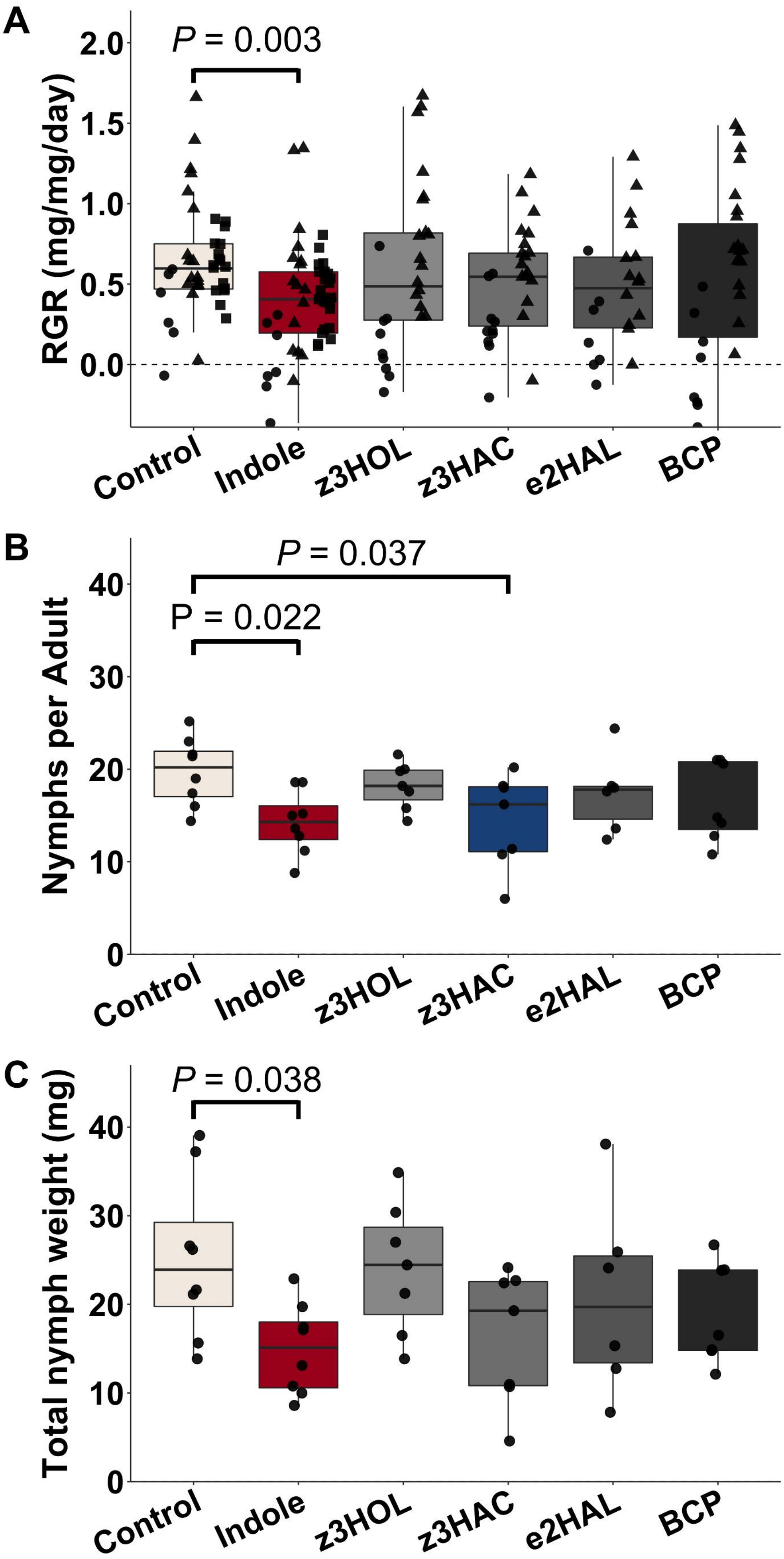
Seed Exposure to z3HAC and z3HOL Enhances M. truncatula Growth. M. truncatula plants grown from seeds exposed to *z*3HAC were noticeably larger than controls (Fig. 3 A). Moreover, using standardized metrics for *M. truncatula* growth (Fig. 3B), *M. truncatula* plants from *z*3HOL- and *z*3HAC-treated seeds had greater petiole length (Fig. 3 C), leaf blade length (Fig. 3D), axillary shoot length (Fig. 3E), and leaf blade width (Supplemental Figure S3) compared to control plants. In contrast, main shoot length was not affected by either *cis*-GLV (Supplemental Figure S4A). Furthermore, while *z*3HOL and *z*3HAC affected the vegetative growth, there was no apparent difference in fruit production of plants grown from volatile-exposed seeds relative to control seeds (Supplemental Figure S4B). No other volatile tested, including indole, affected vegetative growth of *M. truncatula*.

Seed Exposure to Indole does not Affect Herbivore-Inducible Defense Gene Expression after Caterpillar or Aphid Herbivory. Since there was a clear effect of indole seed treatment on caterpillar and aphid fecundity, we assessed whether this effect was due to indole-mediated changes in inducible responses. In *A. thaliana* challenged with *S. exigua*, we analyzed the expression of key marker genes related to JA synthesis (*LOX2*, Fig. 4 A) and signaling (*MYC2* and *VSP2*, Fig. 4B,C), and myrosinase (TGG1, Fig. 4D) and indolyl glucosinolate biosynthesis (*CYB79-B2* and *CYB79-B3*, Fig. 4E,F) important for defense against chewing herbivores in Arabidopsis (Dombrecht et al. 2007; Mewis et al. 2006). JA synthesis and signaling pathways are known to be primable by HIPVs (Arimura et al. 2000; Engelberth et al. 2007; Frost et al. 2008c). Caterpillar herbivory induced the expression of these six marker genes as expected, whereas indole-seed treatment neither directly stimulated nor statistically altered the caterpillar-induced expression patterns of these genes. In *M. truncatula* challenged with aphids, we analyzed two SA-regulated marker genes, *PR5* and *BGL-1*, which have previously been shown to be responsive to aphid feeding (Gao et al. 2008; Maurya et al. 2018; Moran and Thompson 2001). *PR5* and *BGL-1* were induced by aphid feeding (Fig. 4G,H), but indole seed treatment neither directly stimulated nor statistically altered the aphid-induced expression patterns of these genes. That is, in all cases, indole did not directly induce or affect the magnitude of herbivore induction of these defense genes at the time points measured.

Discussion

We show that post-dormancy, ungerminated ("activated") seeds are viable receivers of individual volatiles in ways that prime resistance and, in some cases, directly stimulate growth. Specifically, our study demonstrates that the pre-germination exposure of seeds to indole enhances resistance against herbivores of two feeding guilds in two different plant species without any apparent effects on plant growth or fitness in a single generation of two plant species. Our study also showed that seed exposure to *z*3HOL and *z*3HAC can enhance plant growth in *M. truncatula*. Biotic cues that reliably indicate future biotic stress can prime plant defenses for faster and/or stronger defenses following subsequent stress events (Conrath et al. 2006; Frost et al. 2008a). The phenomenon of HIPV-mediated priming is now well established in aboveground plant organs (Engelberth et al. 2004; Erb et al. 2015; Frost et al. 2007; Frost et al. 2008b; Frost et al. 2008c; Rodriguez-Saona et al. 2009). To our knowledge, our study is the first to show that seeds can also be primed by common components of HIPV blends. Moreover, seed exposure to indole and *z*3HAC at the concentrations we used had no adverse effect on seed germination, vegetative growth and reproductive output of the primed plants (Figs. 2 and 3). Such a long-persisting defense response

Fig. 1 Seed exposure to indole reduces herbivore performance. The effect of seed exposure to plant volatiles on the herbivore fitness (A) Relative growth rate (RGR) of *Spodoptera exigua* caterpillars after 24 h herbivory on *Arabidopsis thaliana* plants grown from control and volatile-exposed seeds. (B) Fecundity (nymphs produced per adult) after 14 days of *Acyrthosiphon pisum* herbivory on *M. truncatula* plant grown from control and volatile-exposed seeds. (C) Total weight of nymphs produced after 14 days. S. exigua growth (A) data were analyzed by mixed models (lme4) with Treatment as a fixed factor and Experimental Trial as a random factor; *A. pisum* fecundity (B) and nymph weight (C) were analyzed by general linear model (glm). All models were subject to Dunnett's post-hoc for pairwise comparisons against the control group. Dots represent individual experimental herbivores, different shapes represent different experimental trials. For the box plots, the center line is the median of the data, the lower and upper hinges correspond to the first and third quartiles, and the whiskers are 1.5* IQR (Interquartile range) in either direction. z3HOL = (*Z*)-3-hexenol; z3HAC = (*Z*)-3-hexenyl acetate; e2HAL = (*E*)-2-hexenal; BCP = β-caryophyllene



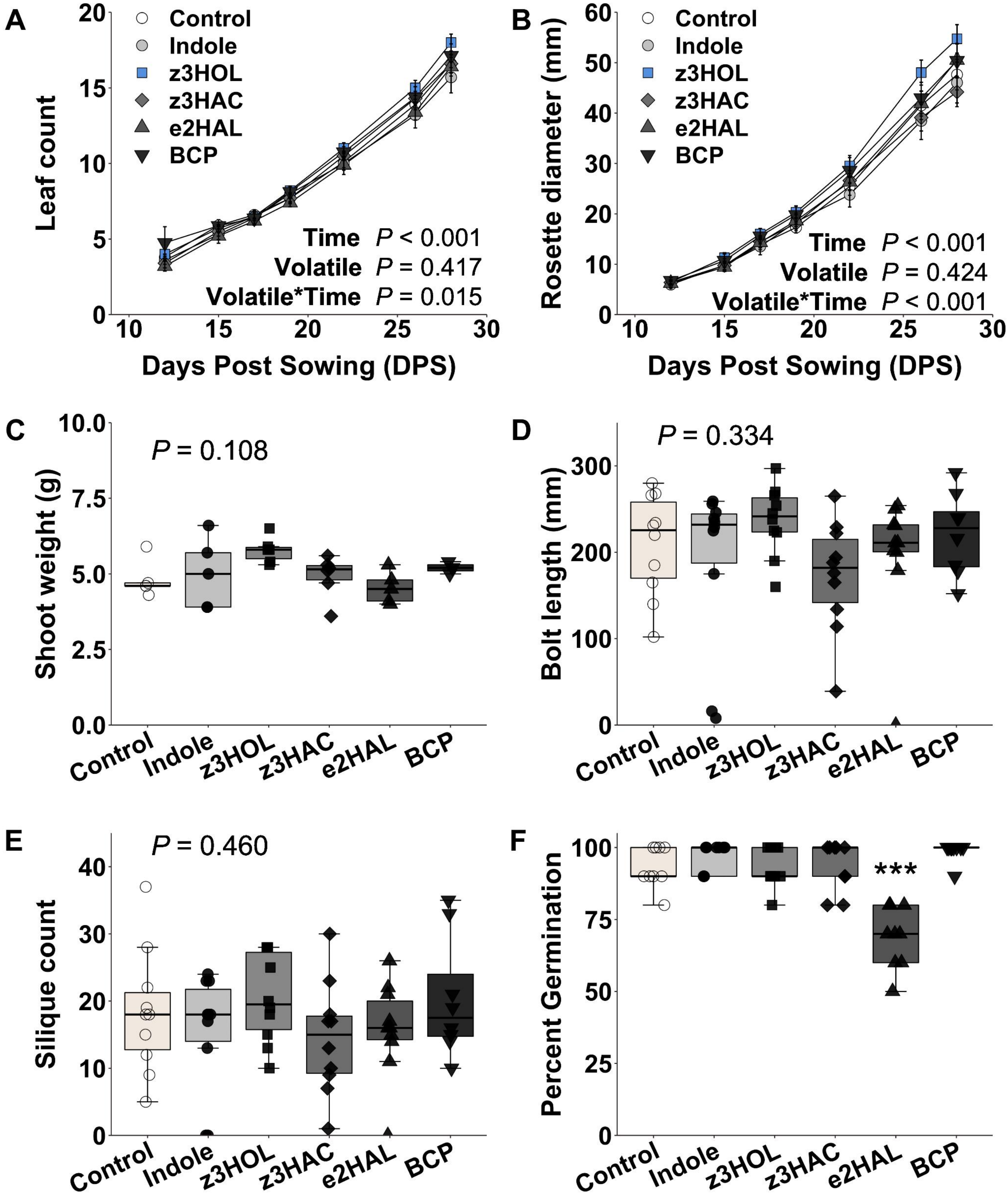


Fig. 2 Seed exposure to plant volatiles does not affect *Arabidopsis thaliana* plants growth and reproductive output. The effect of seed exposure to plant derived volatiles on (A) the number of leaves produced, (B) rosette diameter, (C) shoot weight, (D) bolt length and (E) silique count. DPS represents days after seed sowing. Points (A,B) are means ± 95% SEM ($n = 8$–10). (F) Seed germination efficiency. Points in B-F represent individual observations, their arrangement is spaced using jitter (ggplot). z3HOL = (*Z*)-3-hexenol; z3HAC = (*Z*)-3-hexenyl acetate; BCP = β-caryophyllene; e2HAL = (*E*)-2-hexenal. *** $P < 0.001$

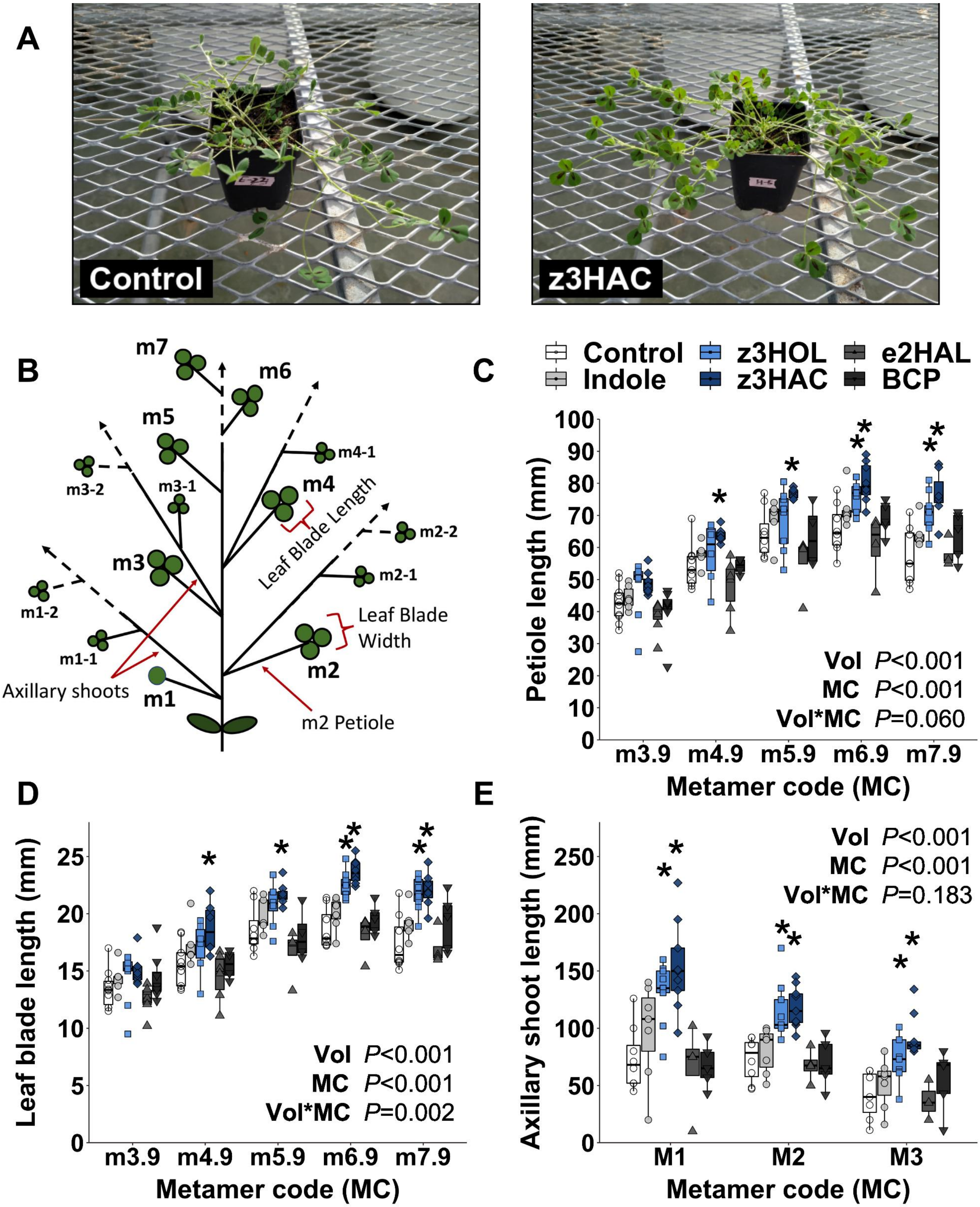


Fig. 3 Seed exposure to *cis* configuration green leaf volatiles enhances growth of *Medicago truncatula*. (A) Picture of representative control and *z*3HAC seed exposed *M. truncatula* plants. (B) Diagram of nomenclature and standardized measurements for *M. truncatula* (Bucciarelli et al. 2006). Nomenclature coding starts with the unifoliate leaf as the first metamer and subsequent trifoliate are labeled along the main shoot in ascending order. Axillary shoots are named as per the metamer of origin. (C) petiole length, (D) leaf blade length, and (E) axillary shoot length of *M. truncatula* plants. "Vol" – Volatile; "MC" – Metamer Code. All the measurements were taken when the leaves were fully developed (i.e., ".9" classification per *M. truncatula* nomenclature (Bucciarelli et al. 2006)). z3HOL = (*Z*)-3-hexenol; z3HAC = (*Z*)-3-hexenyl acetate; BCP = β-caryophyllene; e2HAL = (*E*)-2-hexenal. * $P < 0.05$ of the volatile treatment group compared to controls based on Dunnett contrasts

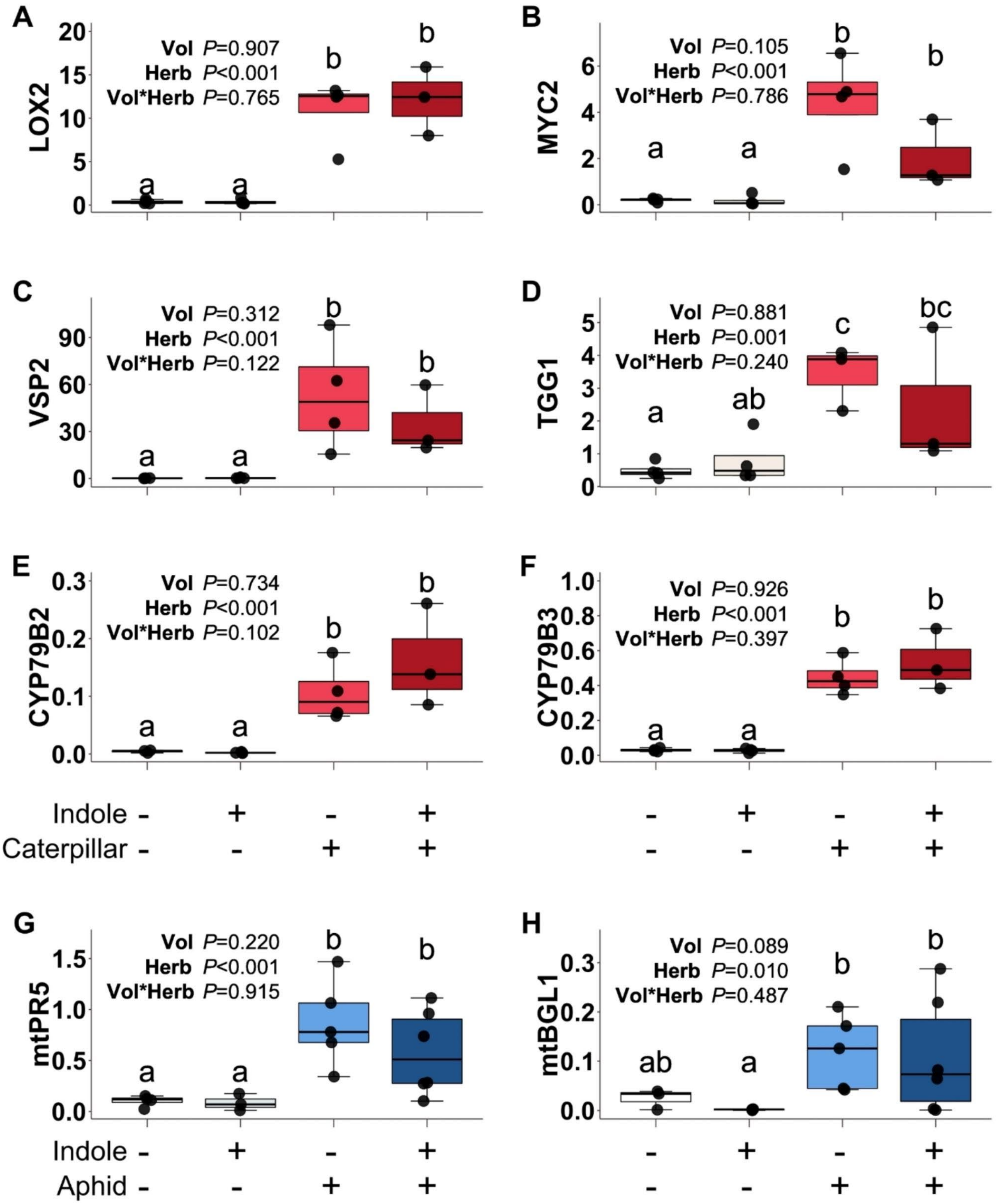


Fig. 4 Seed treatment with indole does not enhance herbivore-induced expression of defense marker genes relative to controls. Relative transcript levels of the genes Lipoxygenase 2 (*LOX2*), *MYC2*, Vegetative Storage Protein 2 (*VSP2*), Myrosinase 1 (*TGG1*), Cytochrome P450 79s (*CYB-B2* and *CYB-B3*) in *A. thaliana* after 24 h of *Spodoptera exigua* herbivory was measured by quantitative RT-PCR analysis (A-D). Similarly, transcript levels of SA regulated marker genes Pathogenesis-Related 5 (*PR5*) and β-Glucosidase 1 (*BGL-1*) were measured in *Medicago trunacatula* after 14 days of *Acyrthosiphon pisum* aphid herbivory (E & F). Relative expression was determined ($2^{-\Delta Ct}$) using the geometric mean of two housekeeping genes for normalization. Dots represent individual biological replicates. Different letters on the bar represent significant difference ($p < 0.05$) based on Tukey HSD contrasts. "Vol" – Volatile; "Herb" – Herbivore

without apparent negative consequence on plant growth and development is indicative of defense priming via direct activation of defense responses, which potentially provides a contrast to priming of leaves by HIPVs.

HIPV-mediated defense priming is theoretically a component of an inducible resistance phenotype (Frost et al. 2008c; Hilker et al. 2015), but this was not the case in our study with synthetic volatile treatments to activated seeds. Since seed treatment with defense phytohormones (e.g., JA, SA and BABA) primes defenses by modulating stress-related signaling pathways (Azooz 2009; Haas et al. 2018; Worrall et al. 2012), we hypothesized that volatile indole would prime seeds through inducible signaling pathways. We therefore predicted that seed-primed plants would show primed inducible defenses compared to controls when challenged with herbivores. For example, Worrall et al. (2012) showed that seed treatment with JA and BABA primed antiherbivore and antipathogen defenses in the subsequent Arabidopsis plants by JA-dependent processes. However, in our case, JA-related octadecanoid pathway (Ballare 2011; Wasternack 2007) and glucosinolate biosynthesis (Hopkins et al. 1998; Reymond et al. 2004) marker genes were induced by *S. exigua* feeding to similar levels independent of indole seed treatment (Fig. 4). Similarly, marker genes for SA-related defense (Walling 2008) in *M. truncatula* were induced by *A. pisum* but were not additionally enhanced by seed treatment (Fig. 4). Given that some of these marker genes (i.e., LOX2 and VSP2 [a MYC-branch marker gene (Vos et al. 2013)]) are primable (Frost et al. 2008c) and MYC2 is a potential regulator of priming of the JA pathway (Dombrecht et al. 2007; Kazan and Manners 2013), volatile-mediated seed priming apparently operates through a mechanism of directly activating resistance. That said, indole seed treatment did not induce any marker gene before herbivory (Fig. 4), further ruling out activation of induced resistance via seed priming and supporting direct activation of resistance. Since we measured just single time points as indicators of inducible defense, it is possible that seed priming altered the temporal dynamics of induced defense, one hallmark of priming (Frost et al. 2008a). However, the lack of transcriptional priming in *A. thaliana* grown from indole-treated seeds is particularly noteworthy because it has been established that transcriptional evidence of priming is observable after 24 h of real caterpillar herbivory (Frost et al. 2008c). Moreover, while individual marker genes may not necessarily reflect priming that might be observed if interrogating at a transcriptome level (Appel et al. 2014; Frost et al. 2008c), at least some of the marker genes we tested in *A. thaliana* are known to be primed by HIPVs or synthetic components of HIPV blends and therefore had a reasonable expectation of serving as "priming" marker genes in this study. In contrast, the interpretation of the *M. truncatula* data are more limited since the single time point at the end of a 14 d feeding trial only indicates the lack of a sustained effect of indole seed treatment on the expression of two aphid-induced genes (Maurya et al. 2018), even though a stronger sustained response is also an important aspect of defense priming (Frost et al. 2008a). Taken together, the enhanced resistance in plants grown from seeds exposed to indole in our study may therefore be a result of the changes in plant nutritive and defense chemistry that are directly stimulated by seed responses to indole.

Indole was the only volatile we tested in which seed exposure primed anti-herbivore plant defenses in both model plants, and did so across two different feeding guilds. Indole is an ubiquitous, inter-kingdom intermediate in critical biochemical pathways (Zhang et al. 2008) and a signaling molecule (Lee et al. 2015). In plants, indole is also a common HIPV that contributes to direct and indirect defenses (Gasmi et al. 2019; Veyrat et al. 2016) and also acts as a defense priming cue (Erb et al. 2015; Ye et al. 2018). That said, rhizosphere bacteria also produce volatile indole, which can modulate plant growth via auxin signaling (Bailly et al. 2014; Bhattacharyya et al. 2015; Blom et al. 2011; Yu and Lee 2013). We tested the *A. thaliana CYP79B2* and *CYP79B3* genes, which encode enzymes that convert tryptophan (Trp) to indole-3-acetaldoxime (IAOx), a rate-determining intermediate in the pathway regulating indolyl glucosinolate biosynthesis (Mewis et al. 2006; Zhao et al. 2002). *CYP79B2* and *CYP79B3* are induced by the pathogen *Erwinia carotovora* (Jones) and are regulated by JA (Brader et al. 2001; Guo et al. 2013). Seed exposure to indole alone did not upregulate either *CYP79* gene, but *S. exigua* feeding induced their expression independent of seed exposure to indole (Fig. 4 C&D). Therefore, the IAOx pathway, which is one potential route for auxin biosynthesis (Zhao et al. 2002), may not be involved in indole-mediated seed priming. There are several potential pathways for the synthesis of indole in plants (Mashiguchi et al. 2011), so our study cannot necessarily rule out that indole-mediated priming of auxin could occur via a non-IAOx pathway. Moreover, it is possible that indole treatment affected JA signaling through cross-talk with auxin signaling pathways (Tyagi et al. 2018) that we did not capture with our targeted gene expression approach. Nevertheless, seed priming with indole produced consistent effects in two different plant species against different feeding guilds of herbivores. This result is encouraging since responses to caterpillars and aphids are often regulated by antagonistic phytohormone pathways (e.g., JA vs. SA) (Mewis et al. 2005). Since JA-mediated defenses can impact SA-mediated activation of pathogen resistance (Thaler et al. 2002), determining the mechanisms of indole seed priming will be important for predicting its positive and negative effects on defenses against different herbivores and pathogens.

Exposure of *M. truncatula* seeds to two GLVs (*z*3HOL and *z*3HAC) stimulated vegetative growth. Our group has observed similar vegetative and reproductive growth stimulation using a low-dose, persistent application of *z*3HAC in field-grown lima bean plants (Freundlich et al. 2021). *z*3HAC-treated lima bean plants were also better defended (Freundlich et al. 2021), consistent with our *M. truncatula* findings here (Figs. 1 and 3). Moreover, *z*3HOL seed treatment stimulated modest but statistically significant growth over time in Arabidopsis (Fig. 1, S2). GLVs are well-established priming cues against biotic stress (Engelberth et al. 2004; Frost et al. 2008c), and volatile communication between plants can alter biomass allocation (Ninkovic 2003). Our results suggest that GLVs can also stimulate plant growth and ostensibly overcome the growth-defense dilemma (Herms and Mattson 1992) in some plant species. *z*3HAC has been shown to stimulate maize growth in some cases (Engelberth and Engelberth 2019). One caveat, though, is that our group also has shown that persistent exposure to *z*3HAC reduces growth in *Capsicum annuum* (Freundlich et al. 2021), therefore the stimulating effect of *cis*-GLVs is not universal. One plausible hypothesis for future consideration is that z3HAC triggers an overcompensation response in lima bean (Garcia and Eubanks 2019; Godschalx et al. 2016).

Synergy and dose-dependency of volatile cues are important aspects of defense priming. Recently, Hu et al. (2019) showed that indole and z3HAC act synergistically to enhance priming and plant defense in maize. In our study, these were the two volatiles that reduced aphid performance on *M. truncatula*. Even though specific mechanisms underlying synergistic effects of volatile exposure are not fully understood, it is possible that we would have seen even stronger seed priming effect in both systems had we incorporated combinatorial treatments. In addition to synergistic effects, the dose of a volatile cue affects plant responses. For example, the dose of an insect-derived volatile cue impacts the amount of damage inflicted by a specialist herbivore (Helms et al. 2017). We specifically held volatile concentration constant in this study, using ecologically relevant concentrations consistent with recent work (Erb et al. 2015), but the potential for dose-dependent seed priming is also an intriguing possibility that merits future work. Moreover, one limitation of this study we did not account for variation in volatility among the individual volatiles tested. While this would not affect the main treatment effects of indole and z3HAC, it is possible that the effective doses were not the same among the volatiles.

As a final point, our results have potential applications for applied chemical ecology of pest control and seed management. Considerable attention has focused on leveraging priming of innate plant immunity (Dervinis et al. 2010; Pichersky and Gershenzon 2002; Pickett and Khan 2016; Song and Ryu 2013; Song et al. 2015), due in part to presumed lower fitness costs of priming based defenses (Buswell et al. 2018; van Hulten et al. 2006). In-field foliar or soil application of these agents can induce plant defenses against herbivores (Bruce et al. 2003; Song and Ryu 2013; War et al. 2011), but can also be prohibitively costly for large-scale application. In contrast, seed treatments are a common method of inoculating crops (Paparella et al. 2015), and direct application of synthetic components of HIPV blends to seeds could provide more viable priming-mediated solution to pest management. Moreover, *M. thaliana* is a close relative of fodder crop alfalfa and improved vegetative growth after seed treatment with GLVs may provide a mechanism for enhancing fodder capacity and rejuvenating soils during crop rotations. Our experiments here focused on ungerminated seeds that had dormancy overcome experimentally to mimic after-ripening. While this is a critical stage for seed germination, future efforts to determine if such priming can enhance plant innate immunity in dormant seeds will also be essential for potential applications in seed management. Likewise, an important future step will be to establish the ecological importance of our results in field experiments with actual HIPVs. Ultimately, volatile-mediated seed priming may represent a novel mechanism in plant-plant interactions affecting plant-herbivore interactions within a generation, impacting trans-generational dynamics on ecological communities, and potentially modulating resilience to environmental variation.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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